Studies on citrate and malate metabolism in Lycopersicon esculentum.

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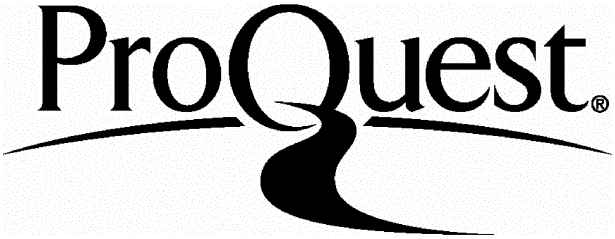

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To Annie.

All those years of impecuniousness were worth it, weren't they luv?

Abbreviations

The majority of the abbreviations used in this thesis are those recommended in the Biochemical Society plublication: Policy of the Journal and Instructions to Authors, Biochemical Journal (1984) 217, pp 1-26.

Non-Standard Abbreviations

MOPS	Morpholinopropanesulphonic acid
BICINE	N, N-bis (hydroxyethyl) glycine
PEG	polyethylene glycol
PVP	Polyvinylpyrrolidone
PPM	Percoll purified mitochondria
TPP	Thiamine pyrophosphate
TEMED	N,N, N', N' Tetramethylethylene Diamine

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Summary

The specific activities of citrate synthase and malate dehydrogenase extracted from mature green fruit of Lycopersicon esculentum, fell 60% during the first two weeks of a twelve week experiment in which the fruit were stored in an atmosphere designed to inhibit ethylene synthesis. Throughout the remainder of the experiment, the specific activities were relatively constant. In the initial two week period, the specific activity of NADP-linked malic enzyme rose by 400%, malic acid concentration fell by 50%, while the concentration of citric acid rose by 20%. Those features of ripening such as the de novo synthesis of lycopene and polygalacturonase, which were thought to depend on ethylene for initiation of response, could not be detected until the fruit were removed to a normal atmosphere. Additionally, citrate synthase and malate dehydrogenase from mature green tomato fruit stored in the presence or absence of ethylene, showed similar trends in specific activity, and the presence of the olefin made no significant difference to the rate of loss of enzyme specific activity.

The purification and partial characterisation of citrate synthase from Lycopersicon esculentum is described. The enzyme is a dimer with sub-units of similar size and a total M_r of approximately 100,000. The characterisation revealed no obvious regulatory features that would easily account for the fall in specific activity.

Sub-cellular fractionation studies demonstrated unequivocally that the site of organic acid metabolism was the mitochondrion. Citrate synthase, NAD-dependent isocitrate dehydrogenase and NAD-dependent malic enzyme were shown to be located exclusively in the

mitochondrion, while malate dehydrogenase was located both in the cytosol and the mitochondrion. All these enzymes including cytosolic malate dehydrogenase exhibited the co-ordinated fall in specific activity described above. A hypothesis is proposed which includes a novel coarse control of the citric acid cycle and related enzymes, as an early indicator of senescence.

INTRODUCTION

Origin, Classification and Anatomy of the Tomato Plant

The subject of this study is both a fruit often classed as a vegetable, and a perennial cultivated in temperate climates as an annual.

The tomato plant is a member of the Solanacea family, belonging to the small genus Lycopersicon. It is indigenous to the western slopes of the Andes and is reputed to have been brought to Europe by Columbus in 1498. The first recorded introduction of the tomato into Europe was by the Italian herbalist P.A. Mattiolus in 1558, quoted by Sturtevant (1889, 1919). The tomato was classified as a member of the Solanacea by Linnaeus (1754) and in the same year Miller separated an esculentum type culture into a genus Lycopersicon.

Essentially all the cultivated forms of the tomato belong to the species Lycopersicon esculentum which has been so extensively cultivated that it ceases to resemble any species found in the natural habitat. It now appears there are no indigenous species outside South America.

The morphological difference between the cultivated and indigenous species is so great that Muller (1940) established two sub-genera. The fruit of sub-genus 1, Eulycopersicon, are glabrous and red or reddish-yellow in colour and contain the species L. esculentum Mill with dubious sub-species L. esculentum var. pyriforme Dun. L. esculentum var. cerasiforme Dun. and pimpinellifolium Mill. The fruit of sub-genus 2, Ericopersicon,

are hairy, white, green or yellowish with lavender or purple stripes. This sub-genus contains the species L. peruvianum Mill, with varieties dentatum Dun. and humifusum Mull., L. cheesmanii Riley from the Galapagos Islands, with the variety minor Mull. L. hirsutum Dun. with the variety glabratum Mull. and L. glandulosum Mull.

As late as 1900 the tomato was still widely regarded as poisonous due to its taxonomic connection with the nightshade family which includes belladonna and mandrake. The toxicity of certain members of the Solanacea is attributable to potent alkaloids. The predominant alkaloid in the tomato is tomatine which is concentrated in the foliage and the green fruit, but which is degraded as the fruit ripen. Even at high concentrations it is much less toxic than the alkaloids present in the nightshades.

Many of the wild tomato species have potential value for agriculture because of the diversity of their germ plasm. Several are resistant to wilt caused by fusarium and verticillium fungus and these qualities have been bred into the cultivated forms. A few wild species exist in quite hostile climatic conditions: L. cheesmanii grows on the shores of the Galapagos Islands approximately two meters above the high-tide line and has been shown to survive in seawater, whereas cultivated forms will die if the concentration of seawater exceeds 50%. Solanum pennellii survives and flourishes in the extremely dry habitat of western Peru, where the main source of water is the fog and mist that penetrates inland from the Pacific. Other useful characteristics of wild tomatoes are insect resistance in L. hirsutum and tolerance of tropical conditions in certain variants of cerasiforme.

The tomato has become a favourite subject for genetic studies for the following reasons:

1. The great wealth of naturally occurring variability in the species which has been supplemented by mutants induced by X-rays and other mutagens.
2. The plant's high rate of self-pollination, leading to the early expression of recessive mutations.
3. The lack of gene duplication within the genome.
4. The plant is easy to grow, has a short life cycle and yields a large number of seeds.

One further asset is the favourable pachytene stage of meiosis, which makes it possible to identify each of the twelve chromosomes of the plant and their arms. Before the advent of genetic engineering, some of the best chromosome maps produced from a flowering plant were obtained from the tomato. The application of the foregoing has resulted in the large-scale production of hybrid tomato cultivars which have increased vigour, earlier ripening and the rapid development of combinations of desirable traits. F_1 hybrids now constitute 95% of the plantings for market tomatoes in Japan and 50% (1978) of those in Israel. The substitution of a number of mutant genes in new cultivars has resulted from the intense breeding activity of the last forty years. One such gene is the (u) or uniform ripening gene, which eliminates the dark green shoulder of unripe fruit and so prevents the retention of chlorophyll in that part of the ripe fruit. The (sp) or self-pruning gene appeared as a spontaneous mutation in Florida in 1914. This mutation causes the plant to grow in an orderly or determinate fashion, in contrast to the usual sprawling indeterminate growth. The branches of (sp)

plants terminate their growth at approximately the same distance from the centre of the plant and the plant flowers more abundantly than the indeterminate type. As a result, fruiting is concentrated in a shorter season and the plants can be harvested by machine (Rick, 1978).

Anatomically, the fruit is a fleshy berry or swollen ovule. The body of the fruit, developed from the ovary wall which surrounds and encloses the seed, is termed the pericarp and consists of outer, radial and inner walls. Locular cavities occur as gaps in the pericarp and contain the seeds embedded in the jelly-like placental tissue. The number of locules varies from two upwards and is characteristic for each cultivar.

Biochemical and Physiological Changes Associated with Ripening

Before discussing any changes associated with ripening, the term itself must be defined.

In experiments designed to measure the respiration rate of apples during the maturation phase, Kidd and West (1930) observed an increase in CO_2 evolution that coincided with visible ripening changes. The authors termed this respiratory pattern the "climacteric" and suggested that it be used as an indicator of ripening. Since then, other interpretations of the climacteric have been put forward. Rhodes (1970) suggested that the term climacteric should be applied to "the whole of the critical phase in the life of the fruit which is triggered by ethylene and during which many changes are occurring". However, McGlasson et al. (1978) drew attention to the confusion in the literature surrounding the term and proposed that the respiratory climacteric be defined as

"the gaseous exchange that accompanies ripening". Biale and Young (1981) referred to the phenomenon as "the physical, chemical, physiological and metabolic changes that are associated with the increase in the rate of respiration, and which occur during the transition phase from the developmental stages of growth and maturation, to the stage of senescence".

The pattern of respiration referred to by Kidd and West (1930) is not universal but is found only in the climacteric fruit, examples of which are apple, banana, breadfruit, peach, pear and tomato. In respiratory experiments with lemon fruit, Biale and Young (1947) noted a steady downward drift in CO_2 evolution for periods as long as six months. Lemon and other fruit such as cherry, grape, lychee and strawberry were termed non-climacteric fruit, since they did not manifest the large respiratory peak, nor the enhanced production of ethylene associated with ripening in the climacteric fruit.

The respiratory time course of climacteric fruit shows a declining trend to a minimum value termed the pre-climacteric minimum, followed by a more or less sharp rise, depending on the species, to the climacteric peak, after which there is a post-climacteric decline in respiration. Fruit softening, colour changes, development of a desirable taste and aromatic flavour are all associated with the climacteric cycle, and the change in development from maturation to ripeness. Although "ripe" has no scientific status (Goodenough and Wright, 1982), it has come to mean a series of both anabolic and catabolic biochemical changes that eventually produce a fruit which is at its most palatable when eaten.

In botanical terms, this is an ideal mechanism for seed dispersal.

The most obvious characteristics of ripening in the case of the tomato are the loss of the green pigment chlorophyll and the appearance of the yellow and red pigments β -carotene and lycopene. As the fruit change from mature green to orange, the respiratory climacteric occurs and CO_2 evolution increases to a peak value twice that of the pre-climacteric minimum (Rhodes, 1980). Concomitantly, ethylene synthesis becomes autocatalytic and ethylene concentration rises from a pre-climacteric minimum of 0.1µl/l to approximately 27µl/l (Lyons and Pratt, 1964). During the colour change from green to orange there is a marked fall in the concentration of malic acid, while citric acid increases up to the orange stage and then gradually declines. Starch hydrolysis is initiated and glucose and fructose concentrations rise although, interestingly, sucrose cannot be detected (de Bruyn et al. 1968). Within forty eight hours the colour changes from orange to red and the fruit begin to soften. The start of softening is correlated with the appearance of the cell wall degrading enzymes polygalacturonase, poly (1,4 - D galacturonide) glyconhydrolase (EC 3.2.1.15) and pectin pectylhydrolase (EC 3.1.1.11). Finally, the fruit becomes very soft to the touch as the internal cellular constituents begin to disintegrate and the seeds eventually mature (Hobson and Davies, 1971).

Exactly when maturation becomes senescence during tomato fruit ripening is not clear. However, accepting Medawar's (1957) definition of senescence as "the deteriorative processes that are natural causes of death", it is safe to assume that senescence will

have started by the breaker/orange stage, since by then irreversible cellular degradation has begun.

The Biochemistry and Regulation of the Ripening Process

Pratt and Goesch (1969) and Rhodes (1970) suggested that the upsurge in respiratory activity reflects the total energy requirements of the synthetic processes associated with ripening. Implicit in this argument is the idea that ripening involves the simultaneous occurrence of a series of interdependent processes. The slow ripening of non-climacteric fruit, such as the orange, would not involve such large energy demands as the climacteric fruit in which ripening occurs over a relatively short period. However, the strawberry, a non-climacteric fruit, can ripen rapidly with major changes in its structure and composition without displaying a respiratory peak (Knee et al., 1977). There is now growing evidence to suggest that ripening consists of a complex co-ordinated series of events, several of which are independent of the respiratory climacteric. Evidence for this as follows:
In the honeydew melon, the process of softening and increased carotenoid synthesis preceded the onset of the respiratory climacteric (Pratt, 1971). The application of cycloheximide to unripe bananas inhibited ethylene production, softening and loss of chlorophyll during storage, without inhibiting the respiratory rise (McGlasson et al., 1971). Dostol and Leopold (1967) demonstrated that gibberellic acid had no effect on the respiratory climacteric of mature green tomatoes but delayed the onset of colour change during storage. The subsequent treatment of such

fruit with ethylene only partially overcame the effects of gibberellic acid. Wang and Mellanthin (1972) showed that 0.08µl/l of ethylene was enough to initiate softening in immature pears without any increase in respiration. These results indicate that it is possible to separate many aspects of ripening from the rise in respiration. Indeed, McGlasson et al. (1971) have expressed the view that the respiratory climacteric is "neither dependent on nor integrated with other aspects of ripening".

Romani (1975) took the view that the respiratory rise is just another independent ripening change relating to the semi-autonomous status of mitochondria within the cell, and he suggested that the respiratory rise is a response to changes occurring in the cytoplasm which either increase the availability of substrates and co-factors, or remove the constraints limiting the in vivo respiratory activity of mitochondria.

It is clear from the foregoing that, in biochemical terms, ripening is a complex interplay of synthetic and degenerative processes. It would be surprising therefore, if the regulation and control of ripening were not itself complex. Yet until relatively recently it was thought that the phytohormone ethylene initiated and controlled the entire process. Ethylene was implicated as a plant growth regulator by Pratt et al., (1948) and has since been shown to regulate several plant processes, ranging from flower senescence to fruit ripening. Its mechanism and regulation of biosynthesis were discovered by Adams and Yang (1981). Ethylene synthesis appears to be constitutive in almost all plant tissues, with the exception of pre-climacteric

fruit (Cameron et al., 1979). Its induction in vegetative tissue is now known to be via stimulation of 1-aminocyclopropane-1-carboxylic acid synthase by indole acetic acid. Ethylene appears to be directly involved in fruit ripening, since exogenously applied ethylene promotes ripening in many climacteric fruit. However, its precise role is still unclear. The rise in ethylene concentration is closely correlated with the respiratory rise, although in some fruit such as the avocado Choquette, banana and the honeydew melon, ethylene synthesis precedes CO_2 production, while in the tomato, plum and the avocado Fuerte the position is reversed.

The effects of ethylene can be simulated by high concentrations of propylene, which has been used to measure the rise of internal ethylene concentration in climacteric fruit. The autocatalytic ability to synthesise ethylene in response to propylene is not universal among climacteric fruit. The tomato seems to be an exception. McGlasson et al. (1975) treated 40-80% mature green tomatoes with propylene and elicited no ethylene production. They concluded that the "onset of ripening in normal tomato is not controlled by endogenous ethylene, although increased ethylene production is probably an integral part of the ripening process". Ethylene reduced the pre-climacteric life of the tomato but even at high concentrations would not induce autocatalytic production. It is almost certain that ethylene is only one of several phytohormones involved in fruit ripening. Kader et al. (1973) showed that externally applied abscisic acid advanced the ripening process in tomato fruit while,

in the pear, indole acetic acid acted both as an inhibitor of ripening, as judged by chlorophyll degradation, and simultaneously promoted ethylene biosynthesis (Frenkel, 1975). The possibility exists that ethylene antagonists are supplied to the fruit via the plant. Supportive evidence for this comes from the work of Gazit and Blumenfeld (1970), who found that the application of 50µl/l of ethylene to avocado Fuerte failed to elicit a ripening response; indeed, if left on the tree the avocado would not ripen. After harvesting, the concentration of internal ethylene in the avocado Choquette rose from 0.04µl/l to 0.1µl/l prior to ripening (Burg and Burg, 1962b). It thus seems that in the avocado the slow disappearance of a factor to a threshold level triggers ripening, rather than a rise in the internal ethylene concentration. In addition, the potentially stimulating levels of ethylene found in apples (Reid et al., 1973) and bananas (Burg and Burg, 1962) are present within the fruit long before ripening.

There is circumstantial evidence which suggests that ethylene may be responsible for the appearance of the cell-softening enzymes. Hobson (1964) has shown that polygalacturonase is present in ripe tomato fruit but cannot be detected in green fruit. The presence of polygalacturonase in ripe tomato fruit has been demonstrated by Tucker and Grierson (1982) to be due to de novo synthesis of enzymic protein. Additionally, Tucker et al., (1980, 1981) have shown the existence of two isoenzymes of polygalacturonase (PG), PG 1 and PG 2. PG 1 has double the molecular mass of PG 2 and, although PG 1 does not reach a very high concentration during ripening, PG 2 increases dramatically.

Evidence for the formation of new mRNA species present in ripe fruit that are not found in immature fruit has been demonstrated by Rattanpanone et al., (1978), although these species have not been shown conclusively to code for PG.

The recent introduction of several ripening mutants of tomato fruit has provided a new tool for examing the regulation of tomato ripening. Although there are mutants which influence tomato fruit pigmentation, the never ripe (nr), ripening inhibitor (rin) and non-ripening (nor) are the only known mutants in which major biochemical changes associated with ripening are either greatly reduced or lacking. The rin mutant can be described as non-climacteric, in that no respiratory burst or ethylene synthesis accompanies ripening. Both the rin and nor mutants have normal pectinesterase activity but only trace amounts of polygalacturonase; in consequence their shelf-life is extremely long. The total carotenoid synthesis is much reduced in both mutants and, in the case of the rin, lycopene is present in trace amounts only (Tigchelaar et al., (1978).

Several methods have been tried to overcome the genetic inhibition of ripening in the rin mutant. In particular, ethylene and propylene were used to induce a temporary climacteric response. However, no autocatalysis of ethylene was observed and in this respect the mutant responds in a typically non-climacteric manner (McGlasson et al., 1975). It is reasonable to assume that, since these fruit fail to respond to exogenously applied ethylene, the lesions do not directly inhibit ripening by blocking ethylene synthesis. In addition, the mutants are capable of producing

ethylene in response to wounding and their methionine levels are similar to those of normal fruit, methionine being the precursor of ethylene (Adams and Yang, 1981).

Mattoo and Vickery (1977) have examined the sub-cellular distribution of isoenzymes (peroxidases, esterases, phosphatases, phosphorylases, malate dehydrogenases and IAA oxidases) in fruits of the cultivars Rutgers and rin. The authors reported that the activity in the supernatant fraction of all these enzymes decreased during ripening of both cultivars, with the exception of malate dehydrogenase which did not differ between genotype or stage of maturity, although no actual data were presented for malate dehydrogenase. The proportion of some enzymes in the particulate fraction, for example peroxidase, indole acetic acid oxidase and several phosphatases, increased during maturation. It is claimed that these findings are consistent with the view that cellular membranes retain their integrity during ripening but that components of metabolism are redistributed between compartments. It is further claimed that enzyme activity would therefore be changed by alteration in sub-cellular distribution and that this constitutes one of the factors controlling ripening.

Hobson (1974) utilised polyacrylamide gel electrophoresis to investigate the isoenzyme pattern in immature, mature, ripe and overripe tomato fruit, cultivar Amberly Cross. Malate dehydrogenase exhibited little change during development, there being four separate isoenzymes. However, several enzymes did show changes during development in their isoenzyme pattern. NADP-linked malic enzyme showed three separate isoenzymes at the mature green

stage, two of which had disappeared by the red stage. Isocitrate dehydrogenase (not stated whether NAD-or NADP-linked) showed a single band at the immature stage which was augmented by a further band from the mature green stage onwards. Hobson also examined large green fruit; these are fruit approximately 10 days prior to the mature green stage. He found that certain enzymes exhibited patterns similar to those found at the mature green stage, for example esterase and 6-phosphogluconate dehydrogenase. However, others such as glutamate dehydrogenase and peroxidase showed patterns characteristic of immature fruit. Hobson suggested that "during development isoenzymes are formed specifically to carry out the changes associated with the final stages of the process". Goodenough and Thomas (1980) examined the electrophoretic pattern of proteins from several different cultivars that had been stored in an atmosphere consisting of 2.5-4% oxygen and 4% carbon dioxide for two months. Although total protein did not decline, a different pattern was observed from fruit removed from the store after two months. This pattern closely resembled that of normal ripe fruit. Apart from the qualitative isoenzyme assessment by Hobson, there has been little or no attempt to determine the activity and control of the enzymes catalysing changes in organic acid metabolism in the tomato.

Retarded Respiration as a Method for Controlling Tomato Ripening

Since the original work of Kidd and West (1933), various atmospheres and temperatures have been used to retard the ripening process. Extremely low oxygen atmospheres have been shown to

prolong the storage of tomatoes and reduce starch degradation (Salunkhe and Wu, 1973). However, the majority of modified atmospheres are normally close to the original one of Kidd and West, i.e. 5% carbon dioxide, 5% oxygen and 90% nitrogen. This combination includes enough oxygen to prevent anoxia occurring, while slowing respiration and ethylene synthesis, since carbon dioxide is a competitive inhibitor of ethylene (Chadwick and Burg, 1967).

Using a modified gas atmosphere, it has been possible to demonstrate a temporal separation between certain biochemical events associated with ripening (Goodenough and Thomas, 1981). In particular, changes in organic acid concentration and starch breakdown can be separated from synthesis of cell softening enzymes and colour change.

Organic Acid Levels During Tomato Ripening

Although originally thought to be synthesised in the leaves and translocated to the fruit, there is now reasonable evidence to support the *in situ* synthesis of citric and malic acids (Buser and Matile, 1977). The predominant acid in ripe tomatoes is citric acid which can reach a concentration of 110meq/100g dry weight (Jeffery *et al.*, 1984). Malic acid is the next abundant acid (Davies, 1964), while other acids such as acetic, formic, lactic and fumaric are present in low concentrations (Bradley, 1960). The three oxoacids— α-oxoglutaric, pyruvic and dihydroxytartaric— are present in canned tomato juice in extremely low concentration (0.0002-0.002%) (Hamdy and Gould, 1962).

Tomato varieties can vary greatly in acidity Simandle et al., (1966) and English varieties grown under comparable conditions differ far more in acidity than in sugar content (Davies and Winsor, 1969). Indeed Davies (1965) suggested that the malic:citric acid ratio was a varietal attribute. In many varieties, total acidity increases during ripening until the breaker stage, followed by a progressive decrease in acidity (Winsor et al., 1962). (Winsor et al., 1962; Sakiyama, 1966) are in agreement that the titratable acidity of the outer pericarp walls is low compared to that of the locular contents. There is now general agreement that malic acid concentration falls during ripening while citric acid increases up to the breaker stage and either falls or exhibits no significant change (Davies, 1966; Carangel et al., 1954). There has been one report that both citric and malic acids increased throughout maturation and ripening; titratable acidity rose until the fruit changed colour and then declined slowly (Dalal et al., 1966). Davies and Winsor (1967) reported that the acidity of the tomato is increased by nitrogen and decreased by phosphate, while Davies (1964) reported that this is specifically true for malic and citric acid concentrations. The form in which nitrogen is applied has a marked effect on the acidity. If nitrogen is applied in the form of ammonia, the acidity is less than if nitrate-nitrogen is applied (Carangal et al., 1954; Sakiyama, 1967). Davies (1964) and Davies and Winsor (1967) reported that high nitrogen, coupled with high potassium, is particularly favourable for high acidity. Calcium and magnesium appear to have little effect on acidity (Bradley, 1962); however, Sakiyama (1966) reported that high potassium

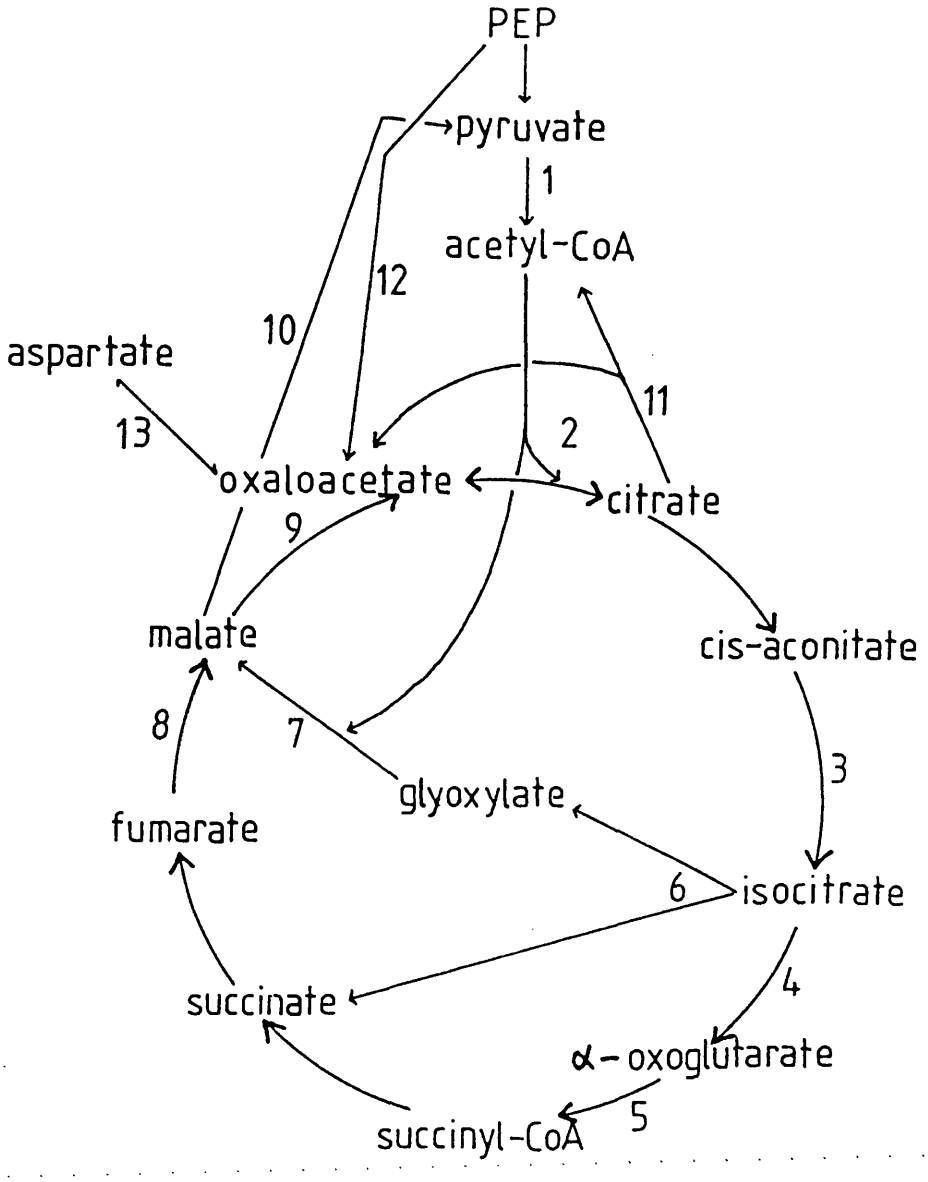
combined with high calcium decreased acidity.

Possible Routes of Citrate and Malate Metabolism

The following scheme (page 17) indicates those reactions involved in the synthesis and utilisation of citrate and malate.

Pyruvate dehydrogenase catalyses the conversion of pyruvate to acetyl-CoA (1). Citrate synthase (2) catalyses the condensation of acetyl-CoA with oxaloacetate to form citrate. Utilisation of citrate can be accomplished via aconitase (3), isocitrate dehydrogenase (4) and α-oxoglutarate dehydrogenase (5). Isocitrate may be split by isocitrate lyase to yield glyoxylate and succinate (6). Reactions (7) and (8), malate synthase and fumarase, catalyse the formation of malate, while its removal is achieved by malate dehydrogenase (9) and malic enzyme (10). Additionally, citrate can be split to give oxaloacetate and acetyl-CoA (11) by ATP-dependent citrate lyase which has been shown to be present in mango fruit (Mattoo and Modi, 1970). Oxaloacetate can be replenished via the anaplerotic reaction catalysed by phosphoenolpyruvate carboxylase (12), in addition to being transaminated to yield aspartate (13).

Exactly where within the cell citrate accumulates is not known, although it has always been assumed that the site of accumulation, if not synthesis, is the vacuole. Many mature fruit contain some of the largest vacuolated cells found in the plant kingdom and this may well be relevant to the storage of high concentrations of organic acids.



Possible routes of citrate and malate metabolism in Lycopersicon esculentum.

Mechanism of Citrate Accumulation in Other Organisms

Reports of citrate accumulation in other organisms are relatively rare. However, under the correct growth conditions Aspergillus niger will excrete citrate into the growth medium, due to a weakly regulated citrate synthase (Kubicek, 1980). Bogin and Wallace (1966) have proposed a mechanism for the accumulation of citrate in Citrus limon compared to Citrus Limettoides. The authors proposed an increased synthesis of citramalate in C. limon compared to C. limettoides due to enhanced CO_2 fixation by phosphoenolpyruvate carboxylase, and high pyruvate amination, thus reducing the pyruvate pool and the formation of the citramalate precursor parapyruvate in C. limettoides. Citramalate is a competitive inhibitor of aconitase and would therefore decrease the utilisation of citrate via the citric acid cycle.

Hirai and Ueno (1977) examined several enzymes of the citric acid cycle from the juice vesicles of ripening Satsuma mandarin and sweet lime. Both these fruit are non-climacteric. In particular, they examined citrate synthase, malate dehydrogenase, malic enzyme, NAD-linked isocitrate dehydrogenase and phosphoenolpyruvate carboxylase. They were able to show four distinct stages during ripening. Stage 1 was marked by rapid increases in all enzyme activities, RNA and protein concentration. During Stage 2, enzyme activities remained constant but the fresh weight increased two-fold and acid accumulation was most prominent during this stage. In Stage 3, the activities of citrate synthase and malate dehydrogenase increased four-fold as did RNA and protein levels, but acid increases were negligible;

this stage was considered the maturation stage. Finally, Stage 4 was considered the senescence stage; NAD-linked isocitrate dehydrogenase activity increased while the activities of phosphoenolpyruvate carboxylase and malic enzyme decreased slightly and RNA and citrate concentrations decreased considerably.

Elias and Givan (1977) demonstrated the presence of NADP-linked isocitrate dehydrogenase in chloroplasts of Pisum sativum. However, citrate synthase could not be detected, neither could the authors determine the source of chloroplastic isocitrate. Since aconitase is not present in chloroplasts, isocitrate is probably metabolised to α-oxoglutarate as a precursor for amino acid synthesis.

Mechanism of Citrate and Malate Accumulation in L. esculentum

Apart from the labelling studies of Wang et al. (1953) there exists little evidence on the mechanism of accumulation of citric and malic acids in L. esculentum. Davies and Maw (1972) injected tomatoes cultivar Craigella with ^{14}C malate and citrate and monitored their metabolism over a 72-hour period. As the fruit changed colour from green to red, the extent of citric acid oxidation to CO_2 dropped to less than half the value found in green fruit. Malic acid, however, was oxidised to CO_2 and converted to citric acid equally in both green and red fruit. With increasing ripeness, aspartic and glutamic acid concentrations increased.

Doyle and Wang (1960) produced evidence from studies using ^{14}C-labelled glyoxylate, implicating malate synthase in the formation of malate. However, these authors could not definitely establish the presence of isocitrate lyase.

Pratt et al. (1965) reported a small increase in the activity of NADP-linked malic enzyme during the climacteric on the vine but no significant increase when the fruit were stored at 15-20°C. NADP-linked isocitrate dehydrogenase activity increased slightly over the climacteric but the overall activity was low.

Swardt and Duvenage (1971) followed the activity of malate dehydrogenase in the pericarp tissue of ripening tomatoes cultivar Beauty. The authors found a 75% fall in the specific activity of malate dehydrogenase between the mature green and red stages. During the same period, there was a 60% fall in the total titrable acidty. It was concluded, "that normal ripening of climacteric fruits is associated with a changeover in metabolic pathways".

Aims of the Investigation

No quantitative enzymological study of citric and malic acid metabolism within the tomato fruit has previously been undertaken. It was therefore proposed to explore the enzymology of these acids. It was planned to investigate enzyme activities at different stages of fruit development and, in particular, those enzymes catalysing the synthesis and degradation of malic and

citric acids, i.e. malate dehydrogenase, citrate synthase, NAD(P)-linked isocitrate dehydrogenase, NAD(P)-linked malic enzyme and pyruvate dehydrogenase would be measured as would the concentrations of the relevant acids. These measurements should define a trend of acid concentration and enzyme activity changes during the ripening period. The use of the gas atmosphere storage facilities at Long Ashton Research Station would be invaluable for these experiments. It was also planned to purify and examine the regulatory properties of those enzymes exhibiting significant changes in specific activity during the ripening period.

Although the site of acid synthesis is assumed to be the mitochondrion, no concrete data are available, nor are there any data concerning the function of other organelles in citric and malic acid metabolism. Sub-cellular fractionation studies of the tomato were therefore undertaken to determine the site of synthesis of these acids and to ascertain if any inter-relationships exist between cellular organelles that could influence the regulation of citric and malic acid metabolism during tomato fruit development.

MATERIALS AND METHODS

Lycopersicon esculentum cv. Sonatine were supplied by Dr. P. Goodenough, Long Ashton Research Station and purchased from Tanyard Nurseries, Lower Weare, Somerset.
cv. Marathon were also supplied by Tanyard Nurseries.
cv. Sarina were supplied by Stourbank Nursery, Wimbourne, Dorset.

Materials Proteins and chemicals used, their source and purity were as follows:-

Acrylamide (specially purified for electrophoresis) - Fisons and B.D.H., Aldolase - Sigma Chemical Co., Amicon Matrex Gel(Red A, Blue A and Orange A) - Amicon Corp., Bio-Rad Protein Assay Kit - Bio-Rad., Carbonic Anhydrase (bovine erythrocytes) - Sigma Chemical Co., Catalase (beef liver) - Boehringer, Chymotrypsinogen A - Boehringer, Citric Acid Determination Kit - Boehringer, Ferritin (horse spleen) - Boehringer, Glyceraldehyde 3-phosphate dehydrogenase - Boehringer, Lactate dehydrogenase (rabbit muscle) - Boehringer, Malate Dehydrogenase (pig heart) - Boehringer, Malic Acid Determination Kit - Boehringer, Morpholinopropanesulphonic Acid - Sigma Chemical Co., N, N - bis(2 - Hydroxyethyl) Glycine - Sigma Chemical Co., N - 2 - Hydroxyethylpiperazine- N' - 2 - Ethanesulphonic Acid - Sigma Chemical Co., Percoll - Pharmacia Fine Chemicals AB., Pyruvate Dehydrogenase (E. coli) - a gift from Dr. M.J.Danson, RNA - Polymerase (E.coli) - Boehringer, Trypsin Inhibitor (soya bean) - Boehringer. Other

general chemicals and sundries were supplied by B.D.H., Fisons, Sigma and Pharmacia.

Buffers

Extraction Buffer

100mM MOPS, 100mM BICINE containing 1% PVP and 3mM EDTA, pH 8.3.

Mitochondrial Extraction Buffer

300mM sucrose, 2mM EDTA, 100mM MOPS, 100mM BICINE, 2mM magnesium sulphate and bovine serum albumin (fatty acid free) at 0.75mg/ml, all adjusted to pH 8.0

Chloroplast Extraction Buffer

300mM sucrose, 2mM EDTA, 100mM MOPS, 100mM BICINE, 1mM manganese chloride, 1mM magnesium chloride, 1mM sodium pyrophosphate, 5mM ascorbic acid and 5mM glutathione all adjusted to pH 8.0.

Percoll Dilution Buffer

230mM sucrose, 5g PEG 8000, 10mM MOPS and bovine serum albumin (fatty acid free) at 0.75mg/ml, all adjusted to pH 7.5.

General Points to Consider When Extracting From Plant Tissue

Plant tissues often contain large quantities of various secondary products, several of which can be utilised as substrates by the ubiquitous phenol oxidases. Plant cells, unlike their animal counterparts, normally contain a thin strip of protoplasm squeezed between the tonoplast and the cell wall; a plant cell therefore consists mainly of non-protoplasmic material of which the vacuolar

contents constitute the majority. Unless precautions are taken when the cell is ruptured, enzymes and secondary metabolites which are normally separated *in vivo* by compartmentalisation mix freely, often with catastrophic results.

The reaction of plant phenolics with proteins fall into two groups: (1) hydrogen bonding - between the hydroxyl group of the phenol and the oxygen atom of the peptide bond; (2) oxidation to quinones, which in turn can oxidise functional protein groups. Quinones are powerful oxidising agents which tend to polymerise; they condense readily by 1,4-addition with the thiol and amino groups of proteins, forming the browning reaction often seen in plant extracts. The plant biochemist now has several means at his disposal to counteract the problems posed by plant secondary products; these include reducing agents such as dithiothrietol, mercaptoethanol and ascorbate which react with any quinones formed by oxidation and effect reduction back to phenols. Polymers such as PVP (both soluble and insoluble) are used to adsorb phenols and quinones. Even so, there is no standard extraction procedure and optimal isolation techniques vary depending on the species of plant under study.

Experimental Treatment of Fruit, Storage Experiment

Four hundred and fifty kilograms of Sonatine were picked at the mature green stage (fully expanded fruit with mature seeds but no loss of green colour), washed at 46°C for 2 min and dried. This procedure helps prevent subsequent fungal attack in the store(Stead and Goodenough, unpublished data). The fruit were placed in a sealed storage chamber containing 6% CO_2, 6% O_2 and 88% N_2. The relative

humidity and temperature were respectively 98% and 12°C. Gas composition was monitored by infra-red analysis for CO_2, and paramagnetic oxygen analysis. The monitoring devices operated solenoid switches connected to CO_2 and N_2 reservoirs and the gas composition was maintained automatically. Breathing apparatus was worn during sampling, to reduce perturbation of the gas atmosphere to a minimum.

Treatment of Fruit in Experiments with or without Ethylene

Ten kilograms of mature green fruit were placed in an unsealed tank (200x200x100cm). The fruit were placed on two metal racks which gave a single layer of fruit on each rack, thus allowing the escape of CO_2 through vents in the bottom of the tank. The tank was connected to an airflow of 50ml/h to provide a slight positive pressure and maintain an ambient atmosphere. Oxygen and carbon dioxide were monitored daily.

A further ten kilograms of fruit were placed in a sealed glass tank containing potassium hydroxide situated below the fruit to remove CO_2; the atmosphere in this tank was supplemented with ethylene to give a final concentration of 27µl/l. This tank was opened daily and fresh ethylene was added after flushing with air. Both tanks were kept at ambient temperature (22°C) and stored in separate rooms.

Preparation of Crude Extracts

All extraction procedures were performed at 0 - 4°C and all glassware and centrifuge rotors were pre-cooled. Washed samples of tomato fruit were cut into small pieces and homogenised at full speed for 15sec

in a Waring blender containing the extraction buffer at a 1:1 w/v ratio. The resulting homogenate was strained through four layers of muslin and spun at 20,000 x g_{max} for 15 min in either a Beckman J21B or a Dupont Sorvall RC-5B centrifuge. The supernatant was used for enzyme assays.

Ethanol Extraction of Citric and Malic Acid

Samples were cut into slices and extracted in boiling ethanol (80%) for 15 min. The solids were then continuously extracted in the same solvent for 24h in a Soxhlet distillation still. The volume of the extract was reduced in a rotary evaporator, made up to 100ml with ethanol and used for citric and malic acid determinations.

Citric Acid Determination

Citric acid concentration was determined using a Boehringer kit consisting of 12 units of citrate lyase, 136 units of malate dehydrogenase, 284 units of lactate dehydrogenase, 6.0mg of NADH and 1.4g of lyophilised glycylglycine buffer pH 7.8. Citrate is cleaved by citrate lyase to yield oxaloacetate and acetate in reaction 1:

1. citrate $\longrightarrow$ oxaloacetate + acetate

in the presence of lactate dehydrogenase and malate dehydrogenase, oxaloacetate and its decarboxylation product pyruvate are reduced to malate and lactate with the concurrent oxidation of NADH in reactions 2 and 3:

2. oxaloacetate + NADH + H^+ $\longrightarrow$ malate + NAD^+

3. pyruvate + NADH + H^+ ⟶ lactate + NAD^+

The amounts of NADH oxidised in reactions 2 and 3 are stoichiometric with the original amount of citrate. The reaction was monitored at 340nm against a blank containing everything except extract. The absorbance was read at t+0 min, citrate lyase was then added and the reaction was allowed to go to completion at t+10 min, when the change in absorbance was noted. Citric acid concentration (c) in g/l was calculated in the following way:

$$c = \frac{V \cdot MW \cdot A}{\varepsilon \cdot d \cdot v \cdot 1000}$$

where V = final vol. (ml)

v = sample vol. (ml)

MW = molecular weight of citrate

d = light path in cm

ε = absorption coefficient of NADH

A = change in absorbance (t+0 - t+10)

Malic Acid Determination

Malic acid was determined using a Boehringer kit consisting of 2400 units of malate dehydrogenase, 160 units of glutamate - oxaloacetate transaminase, 440mg of L - glutamate and 30ml of glycylglycine buffer pH 10. The reaction proceeds as follows:

1. malate + NAD^+ ⟶ oxaloacetate + NADH + H^+

2. oxaloacetate + glutamate ⟶ aspartate + α-oxoglutarate.

The equilibrium of reaction 1 is in favour of malate formation but the removal of oxaloacetate from the system by reaction 2 displaces the equilibrium in favour of oxaloacetate. The amount of NADH formed is stoichiometric with the original amount of malate. The reaction was monitored at 340nm against a blank containing everything except extract; the malate concentration was calculated as for citrate, above.

Chlorophyll Estimation

Chlorophyll was estimated by the method of Arnon (1949). Chlorophyll suspension (0.5ml) was added to 4.5ml of distilled water and 20ml of acetone. The solution was stirred for 10min and then spun at 15,000 x g_{av} for 10min. The supernatant was used for chlorophyll estimation. Chlorophyll concentration (c) in mg/ml was calculated from the following:

$$C = 20.2D_{645} + 8.02D_{663} \times \frac{\text{dilution factor}}{1000 \times \text{initial vol.}}$$

where D_{645} and D_{663} are the absorbances at 645 and 663nm respectively.

Protein Estimation

Protein was measured by the method of Bradford (1976). To 100µl of extract were added 5ml of suitably diluted Bio-Rad reagent. The solution was mixed gently and the absorbance read at 595nm against an appropriate blank. Protein was estimated from a standard curve using thyroglobulin as standard.

Enzyme Assays

Citrate Synthase (EC 4.1.3.7)

Citrate synthase was assayed by the method of Srere et at. (1963). The assay mixture contained the following: 50mM MOPS, pH 7.9, 0.2mM acetyl-CoA, 0.1mM DTNB and 50µl of extract. The reaction was started by the addition of 0.2mM oxaloacetate to make a total volume of 1.0ml. The change in absorbance was followed at 412nm with either a Perkin Elmer 504 or a Pye Unicam SP1800 spectrophotometer at 30°C. A molar absorption coefficient of 13.6×10^3 $l \cdot mol^{-1} \cdot cm^{-1}$ was used to calculate the activity. One unit of activity is that amount of enzyme catalysing the liberation of 1.0µmole of CoA per min under assay conditions.

Malate Dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase was assayed spectrophotometrically. The reaction mixture contained the following: 50mM MOPS, pH 7.9, 0.4mM NADH and 10µl of extract. The reaction was started by the addition of 0.2mM oxaloacetate to make a final volume of 1.0ml. The oxidation of NADH was recorded continuously at 340nm and a molar absorption coefficient of 6.22×10^3 $l \cdot mol^{-1} \cdot cm^{-1}$ was used to calculate the activity. One unit of activity is that amount of enzyme catalysing the oxidation of 1.0µmole of NADH per min under assay conditions.

NADP-linked Malic Enzyme (EC 1.1.1.40)

Malic enzyme was estimated in a reaction mixture containing 50mM MOPS

pH 7.0, 5mM malate, 0.5mM $NADP^+$ and 5mM Mn^{2+}. The reaction was started by the addition of 100µl of extract to make a final volume of 3.0ml, and the absorbance was measured continuously at 340nm. One unit of activity is that amount of enzyme catalysing the reduction of 1µmole of $NADP^+$ per min under assay conditions.

NAD-linked Malic Enzyme (EC 1.1.1.38)

This enzyme was assayed as above, except that the reaction mixture was contained in 1.0ml and the cofactor employed was NAD^+.

NAD-linked Isocitrate Dehydrogenase (EC 1.1.1.41)

Isocitrate dehydrogenase was assayed by a modification of the method of Cox and Davies (1967). The reaction mixture contained the following:

50mM MOPS, pH 7.6, 0.4mM NAD^+, 5mM Mn^{2+} and 50µl of extract.

The reaction was started by the addition of DL-isocitrate to a final concentration of 2mM in a total volume of 1ml. A molar absorption coefficient of 6.22×10^3 $l \cdot mol^{-1} \cdot cm^{-1}$ was used to calculate the activity. One unit of activity is defined as that amount of enzyme which catalyses the reduction of 1µmole of NAD^+ per min under assay conditions.

NADP-linked Isocitrate Dehydrogenase (EC 1.1.1.42)

This enzyme was assayed as above except that the cofactors employed were Mg^{2+} and $NADP^+$.

Lactate Dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase was assayed spectophotometrically. The reaction mixture contained the following: 20mM tris-HCl, pH 8.0, 0.2mM sodium pyruvate, 0.2mM NADH. The reaction was started by the addition of 30µl of extract to give a final volume of 1.0ml. The oxidation of NADH was recorded continuously at 340nm and a molar absorption coefficient of 6.22×10^3 $l \cdot mol^{-1} \cdot cm^{-1}$ was used to calculate the activity. One unit of activity is that amount of enzyme catalysing the oxidation of 1µmole of NADH per min under assay conditions.

NADP-linked Glyceraldehyde 3 - Phosphate Dehydrogenase (EC 1.2.1.9)

This enzyme was assayed by the method of Jackson et al. (1979). The reaction mixture (3.0ml) contained the following: 67mM MOPS, pH 7.3, 3.3mM ATP(di-sodium salt), 10mM magnesium chloride, 4mM EDTA, 139µM NADPH, 3.3µg/ml of phosphoglycerate kinase and 1mM DTT. The extract was incubated in the above mixture to allow activation and the reaction was started by the addition of 3 - phosphoglycerate to a final concentration of 1mM. One unit of activity is that amount of enzyme catalysing the oxidation of 1µmole of NADPH per min under assay conditions.

Glycollate Oxidase (EC 1.1.3.1)

Glycollate oxidase was assayed by the method of Feieraband and Beevers (1972). The reaction mixture (3.0ml) contained the following: 33mM triethanolamine-HCl, pH 7.8, 2.7mM EDTA, 0.67mM

oxidised glutathione, 0.2mM flavin mononucleotide, 3.3mM phenylhydrazine-HCl, pH 6.8, and extract. The reaction was started by the addition of glycollic acid to a final concentration of 5mM. The assay was followed continuously at 324nm and a molar absorption coefficient of 1.7×10^4 $l \cdot mol^{-1} \cdot cm^{-1}$ was used to calculate activity. One unit of activity is that amount of enzyme catalysing the formation of 1μmole of glyoxylate phenylhydrazone per min under assay conditions.

Succinate Dehydrogenase (EC 1.3.99.1)

Succinate dehydrogenase was measured by the method of Muller et al. (1968). The reaction mixture (1.0ml) contained the following: 2mM dichlorophenolindophenol, 30mM phenazine methyl sulphonate, 15mM potassium cyanide, 100mM MOPS, pH 7.6, and extract. The reaction was initiated by the addition of neutralised succinic acid to a final concentration of 6mM. The assay was followed continuously at 600nm and a molar absorption coefficient of 1.61×10^4 $l \cdot mol^{-1} \cdot cm^{-1}$ was used to calculate activity. One unit of activity is that amount of enzyme catalysing the reduction of 1μmole of dichlorophenolindophenol per min under assay conditions.

Pyruvate and α-Oxoglutarate Dehydrogenase

(EC 1.2.4.1 and EC 1.2.4.2)

Solution A contained the following: 40mg NAD^+, 5.3mg of TPP, 11.6mg of $MgCl_2$ and 14.3ml of 200mM potassium phosphate, pH 8.0, in 40ml of distilled water.

Solution B contained the following: 11.7mg of CoA and 41mg of

cysteine-HCl in 20ml of distilled water.

Solution C contained either 1.123g of sodium pyruvate or 1.68g of monosodium α-oxoglutarate in 100ml of distilled water. The reaction mixture consisted of: 900µl of solution A, 20µl of solution B and 60µl of extract. The reaction was started by the addition of 20µl of solution C and the reaction was followed continuously at 340nm. One unit of activity is that amount of enzyme catalysing the reduction of 1µmole of NAD^+ per min under assay conditions.

Molecular Weight Estimation by Gel Filtration

Sephacryl S-200 was poured under gravity to fill a glass column (100 x 1.5cm). When packed, the column was equilibrated with 3l of 50mM MOPS/BICINE, pH 7.8, at a flow rate of 5ml/h. Proteins were pumped onto the column in a total volume of 1ml of the elution buffer and 1ml fractions were collected.

Calculation of Void Volume

The void volume was determined by running blue dextran separately at a concentration of 1mg/ml; the molecular weight of blue dextran is in excess of 1×10^6 and is completely excluded from the gel.

Electrophoresis

SDS Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis was used to estimate the sub-unit M_r of citrate synthase and malate dehydrogenase. 7.5% gels consisted

of the following:

12.7ml acrylamide/bisacrylamide (30:0.8g/100ml)

27.4ml double distilled water

10ml of 1.86M tris-HCl buffer, pH 8.8

25µl of TEMED

30mg of ammonium persulphate

The bridge buffer consisted of 6.05g tris, 28.8g glycine, plus 20ml of 10% SDS made up to two litres, pH 8.3. Marker proteins and samples were boiled for a minimum of two minutes in an equal volume of the following dissolving buffer:

0.5ml of 1.86M tris-HCl buffer, pH 8.8

2.5ml of 10% SDS

1.25ml of glycerol

0.62ml of 2-mercaptoethanol

8.12ml of double distilled water

0.001% bromophenol blue

Gels were run at 3mA per tube until the bromophenol front was approximately 0.5cm from the bottom of the tube.

Non-Denaturing Polyacrylamide Gel Electrophoresis

Slab non-denaturing polyacrylamide gels were run using an LKB apparatus and consisted of a stacking and resolving gel.

Resolving Gel.

Sol. 1. 50ml of 1M HCl, 36.6g of tris and 0.23ml of TEMED in 100ml of distilled water.

Sol. 2. 60mg of acrylamide and 1.6g of bisacrylamide in 100ml of distilled water.

Sol. 3. 0.14g of ammonium persulphate in 100ml of distilled water.

The gel mix consisted of: 5ml of sol. 1, 10ml of sol. 2, 20ml of sol. 3 and 8ml of distilled water. This solution was injected into the gel mould using a 50ml syringe and overlayed with water.

Stacking Gel.

Sol. 4. 48ml of 1M KOH, 2.87ml of acetic acid and 0.46ml of TEMED in 100ml of distilled water.

Sol. 5. 10g of acrylamide and 2.5g of bisacrylamide in 100ml of distilled water.

Sol. 6. 0.004g of riboflavin in 100ml of distilled water.

The gel mix consisted of: 1ml of sol. 4, 2ml of sol. 5, 1ml of sol. 6 and 3.5ml of 50% sucrose in distilled water. This gel was set by UV radiation after being layered onto the resolving gel. The gel was run at 2mA per track until the proteins had traversed the stacking gel, when the current was increased to 4mA per track. Gels were stained in the following solution: 25% isopropanol, 10% acetic acid and 0.05% coomassie blue, and destained in 10% acetic acid. Alternatively, gels were stained using the silver stain (Morrisey, 1981).

Preparation of Material for Sub-Cellular Separation

Tomato fruit were quartered and the placental tissue removed with a scalpel. The quartered pericarp was gently grated below the surface of the mitochondrial extraction buffer using a cheese grater (Hobson, 1969). The buffer to tissue ratio was 3:1 and a minimum of 600ml of buffer was needed to allow adequate grating. Ascorbate, to a final

concentration of 30mM, was added just before extraction. The resulting slurry was strained through eight layers of muslin and spun at 2,500 x g_{max} for 10min. The supernatant was spun at 10,000 x g_{max} for 30min and the resulting pellet was resuspended in a vast excess (approx. 300ml) of mitochondrial extraction buffer. The resuspended pellet was spun at 900 x g_{max} for 5min and the resulting supernatant was centrifuged at 10,000 x g_{max} for 30min. The crude mitochondrial pellet was gently resuspended in approximately 6.0ml of Percoll dilution buffer and half this volume was used to load the gradients. The intermediate pellets and supernatant were retained for analysis, to allow the construction of a balance sheet of enzyme activity.

Sub-Cellular Localisation Using Percoll Gradients

In Situ Gradients

Percoll was made iso-osmotic, by diluting 9 parts of Percoll with 1 part of 2.5M sucrose. A model experiment was then performed to standardise conditions. Iso-osmotic Percoll was diluted with Percoll dilution buffer in ratios from 1+9 to 9+1, in a final volume of 30ml. Percoll internal density marker beads were loaded onto the top of the gradient, the gradients were then inverted several times to mix the contents and spun. The internal markers (which are dextran beads) reach their equilibrium position and a graph of buoyant density against Rf values for the beads can be plotted. From this, the gradient with the most suitable profile can be chosen.

Discontinous Pre-Formed Gradients

Gradients were prepared by a modification of the method of Jackson et al. (1979). Iso-osmotic Percoll was diluted with Percoll dilution buffer to give the required percentage discontinuities; these were carefully layered ontc each other using a Pasteur pipette and the completed gradients were stored at $4^{o}C$.

Continuous Pre-Formed Gradients

Continuous Percoll gradients were prepared by the method of Takabe et al. (1979). Using an LKB gradient mixer and peristaltic pump, 30ml gradients were pumped at a flow rate of 1.5ml/min into thin walled Dupont polyallomer tubes.

Gradient Fractionation

Gradients were initially fractionated by sucking the contents out from the bottom, through a tube that was passed carefully into the gradient. However, this proved to be ineffective as considerable mixing occurred. Eventually, thin wall 50ml polyallomer tubes were purchased from Dupont and the gradients were eluted from the bottom, using a MSE gradient fractionater. This consisted of a device which clamped the tube with an air tight seal at the top. A hollow needle was threaded up from underneath and pierced the bottom of the tube; air was allowed into the system via a finely controlled bleed mechanism at the top of the tube, thus allowing the speed of fractionation to be controlled.

Estimation of Protein in Gradient Fractions

Since the gradient constituents contained BSA and the concentration of BSA varied throughout the gradient, it was imperative that the correct blank was used. The blank consisted of an identical gradient that was loaded with 3ml of Percoll dilution buffer. This gradient was fractionated in an identical manner to the experimental gradient and aliquots from each fraction were used as blanks for the corresponding fraction from the experimental gradient.

Criteria for Establishing Mitochondrial Integrity

The physical and respiratory integrity of washed and purified mitochondria was determined by two methods:

1. Latency of enzyme activity with and without 0.03% Triton in the assay mixture.

2. Use of the oxygen electrode to calculate respiratory control and ADP : O ratios. Furthermore, washed and purified Percoll mitochondria were scanned continuously between 700 and 300nm to illustrate the difference in chlorophyll and carotenoid contamination between them. Mitochondrial respiration was measured polarographically using a Clark type oxygen electrode. Washed and PPM were injected carefully into the reaction chamber of the oxygen electrode in the presence of 2ml of mitochondrial reaction buffer that had been allowed to equilibrate at 30°C. The reaction buffer consisted of the following: 300mM sucrose, 10mM potassium dihydrogen phosphate, 10mM MOPS, 5mM magnesium chloride and 0.5mM EDTA, pH 7.3. After 1min, 100µl of malate, pH 7.3, (final

concentration 17mM) were added. Once the recorder had stabilised, 100µl of ADP, pH 7.3, (final concentration 100µM) were added. Further aliquots of ADP were added as necessary.

Citrate Synthase Purification Procedures

DEAE-Sephacel

Several kilograms of Sonatine (from 2.5 to 5.0) were extracted as described; the resulting supernatants were combined and subjected to ammonium sulphate fractionation at 50% and 80% saturation. The pellet from the 80% fraction was resuspended in a minimum of 20mM tris-HCl, pH 8.0, and dialysed against 5l of the same buffer overnight. After dialysis, the solution was spun at 20,000 xg_{max} for 5min to remove any precipitate, and loaded onto a DEAE-Sephacel column (1.5 x 30cm) which had previously been equilibrated with 20mM tris-HCl, pH 8.0. The column was then washed with the same buffer until the A_{280} of the eluant was below 0.1. The column was eluted with a linear potassium chloride gradient between 0 - 500mM in 20mM tris-HCl, pH 8.0, at a flow rate of 20ml/h. Fractions (2ml) were collected and assayed for citrate synthase activity.

Use of Dyematrex Kit in Citrate Synthase Binding Study

Dye matrex gels consist of industrial dyes covalently linked to agarose and have been used with considerable success in the purification of certain classes of enzymes, especially the dehydrogenase (Amicon 1980). However, it is still uncertain how and why binding interactions occur. Dye matrex kits are supplied

with the following gels: Red A, Blue A, Blue B, Orange A and Green A. The gels were prepared by washing in 6M urea and 0.5M sodium hydroxide to remove any free dye, with the exception of Orange A which was washed in 6M urea only, since sodium hydroxide hydrolyses the amide bond in this dye. All the columns (0.5 x 10cm) were equilibrated in the running buffer (50mM MOPS, pH 7.8). 1.0ml of supernatant containing approximately 1.0mg of protein and 1.0 unit of activity was loaded onto each column and allowed to enter the gel. The gels were then washed with the running buffer to remove any unbound protein. Finally, the gels were eluted with either 0.5M potassium chloride or 100µM oxaloacetate + 100µM CoA in 50mM MOPS, pH 7.8.

Amicon Red A Gel Procedure

The extraction procedure was identical to the above. However, after dialysis the 80% fraction was loaded onto a column (1.5 x 30cm) containing Amicon Red A Gel previously equilibrated in 50mM MOPS, pH 7.8, containing 50mM potassium chloride. The column was eluted with a linear potassium chloride gradient from 50-500mM in 50mM MOPS, pH 7.8. Fractions (2ml) were collected and assayed for citrate synthase activity and protein. Fractions with a specific activity greater than 10 were pooled and concentrated by ammonium sulphate precipitation. After centrifugation, the pellet was resuspended in 1.5ml of 50mM MOPS, pH 7.8, containing 50mM potassium chloride and applied to a column of Sephacryl S-200 (1.5 x 100cm) previously equilibrated with the running buffer. The column was eluted with

the same buffer at a flow rate of 5.0ml/h and 0.5ml fractions were collected. Fractions were assayed for citrate synthase activity and the purity of the enzyme was determined by running aliquots from the peak tubes on SDS polyacrylamide gels.

Purification of Citrate Synthase Using ATP-Sepharose

ATP was bound to 4g of Sepharose 4B - adipic acid dihydrazide by the method of Gilham (1971). 1.8mmole of ATP were dissolved in 27ml of distilled water and the pH adjusted to 8.2. All subsequent operations were performed at 0°C. 1.98mmole of sodium periodate were added and after 30min the solution was mixed with 27ml of 1M sodium chloride - 0.2M Tricine, pH 8.2, and 4g of Sepharose 4B - adipic acid dihydrazide that had been prepared by washing in 0.5M sodium chloride and 0.1M Tricine, pH 8.2. The mixture was allowed to stand for 1h with occasional agitation and then treated with 24ml of 0.3M sodium borohydride - 0.2M tricine, pH 8.2. After 1h, a further 1ml of this solution was added, and after standing for another hour the slurry was extracted with 4M sodium chloride to remove unbound material. The combined washings were assayed spectrophotometrically against a control reaction in which no periodate was added, to determine the amount of ATP bound to the Sepharose.

Purification of Citrate Synthase and Malate Dehydrogenase Using Iso-Electric Focussing

An LKB 110ml iso-electric focussing column was used for all

experiments. The anode solution, which was located at the bottom of the column, consisted of the following:

0.5ml of ortho-phosphoric acid

9.0ml of distilled water

20.0ml of 50% sucrose

The gradient consisted of a mixture of light and dense solutions which were pumped into the column from the gradient mixer. The protein sample was incorporated into the light solution. The light and dense solutions consisted of the following:

Dense solution 0.75ml of ampholytes pH 3.5-10 and pH 4-6

40.0ml of sucrose

15.0ml of distilled water

Light solution 0.35ml of ampholytes pH 3.5-10 and pH 4-6

12.0ml of sucrose

43.0ml of distilled water

The cathode solution consisted of: 0.5ml of ethanolamine

19.5ml of distilled water

and was pumped slowly onto the top of the gradient. The column had been pre-cooled via a water jacket fitted around the apparatus. 500V were initially applied to the column; the voltage was then gradually increased to a maximum of 2000V. The column was focussed when the current had fallen to a minimum and stabilised. This could range from 36 - 72h depending on the concentration of protein in the sample and the range of ampholytes used. Fractions were collected from the bottom of the column and assayed for pH and activity.

Separation of Malate Dehydrogenase from Lactate Dehydrogenase Using Fast Protein Liquid Chromatography

Sixty μg of lactate dehydrogenase were added to 8ml of 20mM bis-tris-propane, pH 7.5. The solution was loaded through a superloop onto a mono Q column that had previously been equilibrated with 10 column volumes of 20mM bis-tris-propane, pH 7.5. The column was eluted with a potassium chloride gradient (see attached programme). The gradient ran from 0 - 500mM potassium chloride; therefore within the programme, 0 = no potassium chloride, 50 = 250mM, and 100 = 500mM potassium chloride. The eluate from the column was monitored at 280nm by a UV light source that was connected to a microprocessor-controlled fraction collector. The sensitivity of the monitoring source was set to 0.5 absorbance unit, which corresponded to full-scale deflection of the attached chart recorder. The fraction collector was set to collect 1ml fractions. However, if the monitoring device detected a peak greater than 10% full scale deflection, then only the volume corresponding to the area under that peak was collected. This ensured that separated proteins were not unnecessarily diluted with excess buffer.

Pharmacia Fine Chemicals

Laboratory record

Nr 4480

Date: 5. 10. 83.

Sample: Boehringer Lactate Dehydrogenase

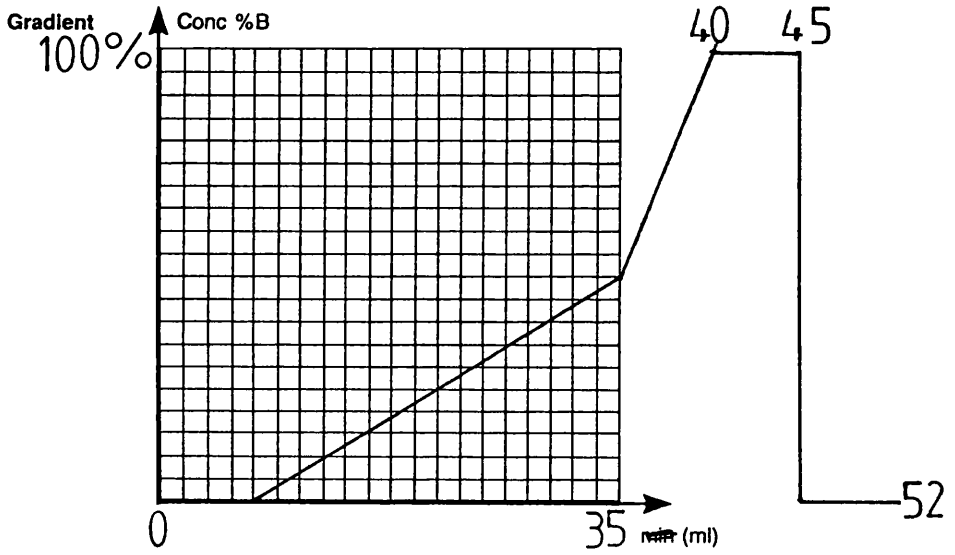
Gel	Column size	Temp
Mono Q	10cm	22 °C

Eluent: Bis.Tris.Propane/HCl and 0-500mM KCl

Flow	Pressure	
1 ml/min ml/min	1.5	~~psi~~ ~~bar~~ MPa

Sample size	
60µg mg/ml µg/µl	8ml µl (ml)

Detector	Sensitivity	Chart speed
280nm	0-0.05	1.0 cm/min (ml) ~~mm/h~~



Method

~~Time~~ (vol) ml	Instruction	Value	Note
0	conc %B	0	
0	ml/min	1	
0	cm/min	1	
0	port set	6.1	port set 6.1 starts the fraction collector.
4	conc %B	0	
35	conc %B	50	
40	conc %B	100	
45	conc %B	100	
45	conc %B	0	
45	port set	6.0	port set 6.0 stops the fraction collector.
52	conc %B	0	

RESULTS

Since it is known that the concentrations of citric and malic acids change as the tomato fruit progresses from maturity to the ripe state (Introduction p.15), a logical starting point for the project was to assay those citric acid cycle enzymes involved in the synthesis and metabolism of citric and malic acid in mature green and ripe fruit, to determine if there was a significant difference in specific activity between the two states.

The pericarp from equal weights of mature green and red tomato fruit, cv. Sonatine were homogenised as described. The resulting supernatant was used to assay for citrate synthase, malate dehydrogenase, NADP- and NAD-linked isocitrate dehydrogenases, pyruvate dehydrogenase, α-oxoglutarate dehydrogenase and NAD-linked malic enzyme. The data indicated a distinct difference in both activity and specific activity between the two states, for citrate synthase, malate dehydrogenase and NADP-linked isocitrate dehydrogenase (Table 1). The specific activities of citrate synthase and malate dehydrogenase fell by approximately 50% between the mature green and red stages, while the specific activity of NADP-linked isocitrate dehydrogenase rose by approximately 50% over the same period. No NAD-dependent isocitrate dehydrogenase activity could be detected at either stage, and the addition of either 0.2mM ADP or 0.2mM AMP, both known activators of eukaryotic and some bacterial isocitrate dehydrogenases Self et al. (1973), had no effect on the rate of oxidation of either $NADP^+$ or NAD^+. Pyruvate dehydrogenase,

Table 1

	Ripe(Red)	Mature Green	Red/Green
citrate synthase	3.67×10^{-2}	6.40×10^{-2}	57%
malate dehydrogenase	2.34×10^{-1}	5.05×10^{-1}	46%
NADP-linked isocitrate dehydrogenase	6.64×10^{-2}	3.50×10^{-2}	189%

All figures are expressed as μmole/min/mg protein.

Table 1. The specific activities of citrate synthase, malate dehydrogenase and NADP-linked isocitrate dehydrogenase extracted from mature green and red Sonatine.

α-oxoglutarate and NAD-linked malic enzyme could not be detected in crude extracts. During all extractions it was noticeable that the supernatants remained quite green and exhibited no signs of "browning". The protein concentration of supernatants from both mature green and red fruit varied between 1.0 - 1.5mg/ml.

Biochemical and Physiological Experiments on Whole Tomato Fruit Stored in a Modified Gas Atmosphere (1)

Previous work by Goodenough and Thomas (1981) demonstrated that, in tomato fruit stored in a modified gas atmosphere, changes in organic acid concentration and starch degradation occur before colour change, and the former changes may be independent of ethylene.

It was therefore decided to utilise the modified gas atmosphere storage facilities at Long Ashton to follow the changes (over several weeks) in the specific activities of citrate synthase, malate dehydrogenase, NADP-linked isocitrate dehydrogenase and NADP-linked malic enzyme, and to attempt to correlate any observed changes with alterations in citric and malic acid concentrations. Additionally, glucose, fructose and ethylene concentrations were monitored by gas chromatography. Furthermore, several features of ripening assumed to be controlled by changes in ethylene concentration were also measured, i.e. the degradation of chlorophyll, the synthesis of lycopene and the changes in specific activities of polygalacturonase and cell wall bound acid invertase (β-fructofuranoside fructohydrolase, EC 3.2.1.26). Mature green Sonatine (450kg) were placed in the store as described. Since whole

fruit were now being extracted, a new buffering system was used as 100mM tris-HCl was not adequate to maintain the pH between 7 - 8. A mixture of 100mM MOPS and 100mM BICINE was used and found to be completely satisfactory, obviating the need for the dropwise addition of concentrated sodium hydroxide during the extraction. Four samples were extracted at weekly intervals and used for protein and enzyme analysis, while a further six separate samples were used for acid determination.

Within two weeks, the specific activity of citrate synthase had fallen to 40% of the starting value. Thereafter, the activity remained constant apart from an apparant decrease between weeks 7 and 8, followed by a return to the original value. After 12 weeks of storage, the fruit were placed in ambient conditions for one week; during this period the specific activity of citrate synthase fell slightly. The citrate content of the fruit rose by 25% during the first 2 weeks of storage, fell by 3% over the next 3 weeks, then rose 10% before falling to a concentration just below the starting level by the end of 12 weeks (Fig.1). The citrate concentration accumulated in the first 5 weeks was less than the concentration of malate lost from the fruit during the same period.

In contrast to the initial rise in citrate, malate concentration fell to 58% of the pre-store level after 2 weeks. Between weeks 2 and 3, the concentration fell a further 3% and thereafter remained relatively constant for the remainder of the experiment until the fruit were removed from the store, when the concentration fell a further 8%.

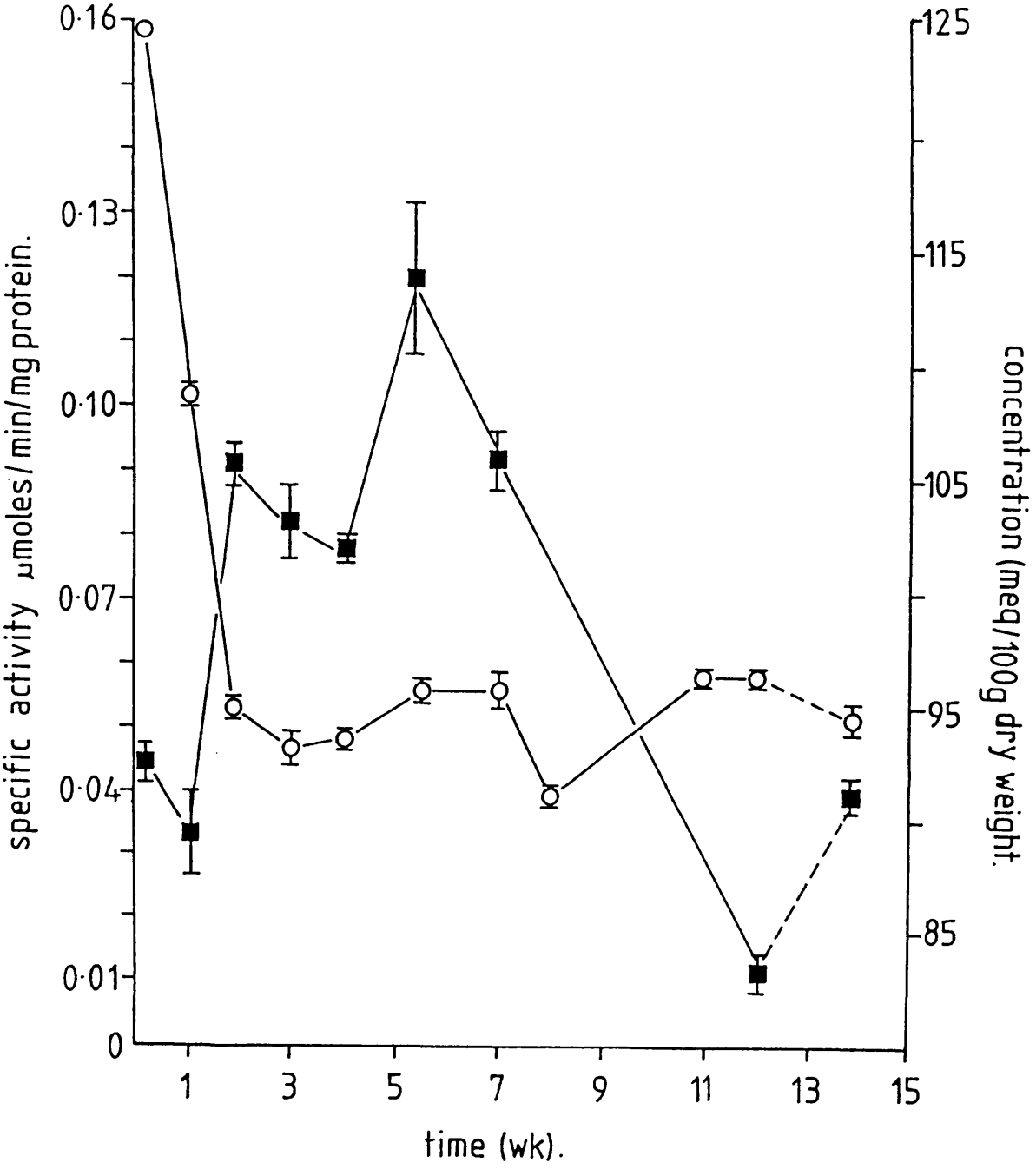


Fig.1 Changes in specific activity of citrate synthase and citrate concentration in stored tomato fruit.

Changes in specific activity of citrate synthase and citrate concentration in tomato fruit cv. Sonatine, held at 12°C in 6% CO_2, 6% O_2 and 88% N_2, and after standing in ambient atmosphere and temperature. Specific activity of citrate synthase during storage(O——O) and in ambient conditions(O- - -O). Concentration of citrate during storage(■——■)and after standing in ambient conditions(■---■). Bars are standard errors of the mean of 4 samples.

The specific activity of NADP-dependent malic enzyme rose during the first 2 weeks of storage by 400%. For the next 8 weeks the specific activity fell slowly with the exception of a small rise between weeks 4 and 5. When the fruit were removed from the store, the specific activity fell to zero (Fig.2).

The specific activity of malate dehydrogenase exhibited a slight rise after one week in store, but then fell to 55% of the starting value by week 3. Thereafter, the specific activity remained constant even when the fruit were removed from the store (Fig.3).

In complete contrast to the rapid changes in specific activities of citrate synthase, malate dehydrogenase and malic enzyme, the specific activity of cell wall bound acid invertase remained at a low level throughout storage, while the specific activity of polygalacturonase was undetectable during the first 7 weeks. However, upon removal from the store, the specific activities of both enzymes rose rapidly within 24h (Fig.4).

After a small rise between weeks 1 and 2, the chlorophyll concentration slowly declined to 50% of its initial value by week 6. However, lycopene could not be detected until after week 7, and remained at a low level throughout. When fruit were removed from the store, chlorophyll decreased rapidly and lycopene accumulated after a 24-48h lag period (Fig.5).

Prior to the fruit entering the store, minute quantities of ethylene were detectable (approx. 0.2ppm). After one week in store, however, ethylene could not be detected and it was not until

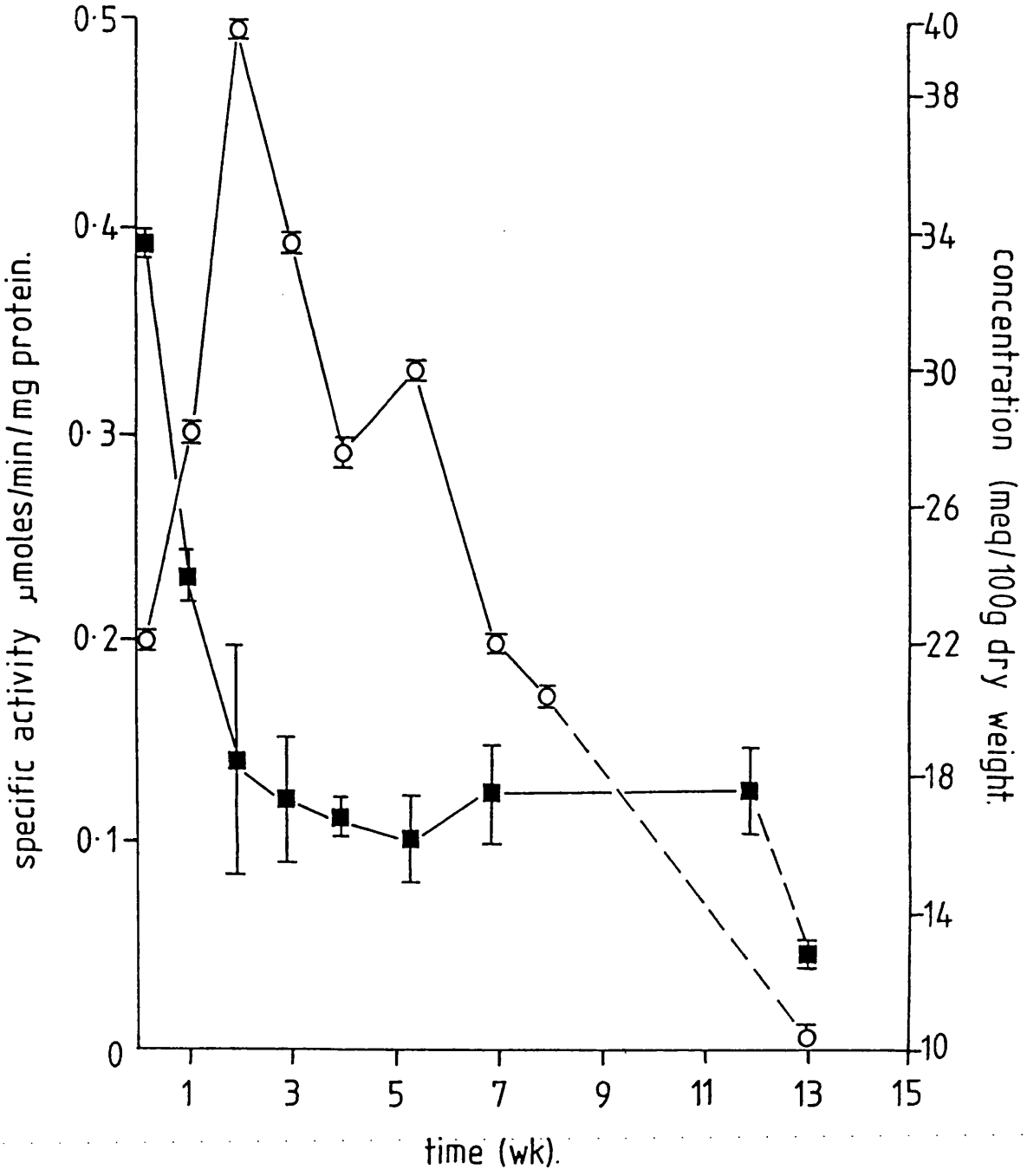


Fig.2 Changes in specific activity of NADP-linked malic enzyme and malate concentration in stored tomato fruit.

Changes in specific activity of NADP-linked malic enzyme and concentration of malate in tomato fruit cv. Sonatine, held at 12°C in 6% CO_2, 6% O_2 and 88% N_2, and after standing in ambient atmosphere and temperature. Specific activity of malic enzyme during storage (O——O) or after standing in ambient conditions (O---O). Concentration of malate during storage (■—■) or after standing in ambient conditions (■---■). Bars are standard errors of the mean of 4 samples.

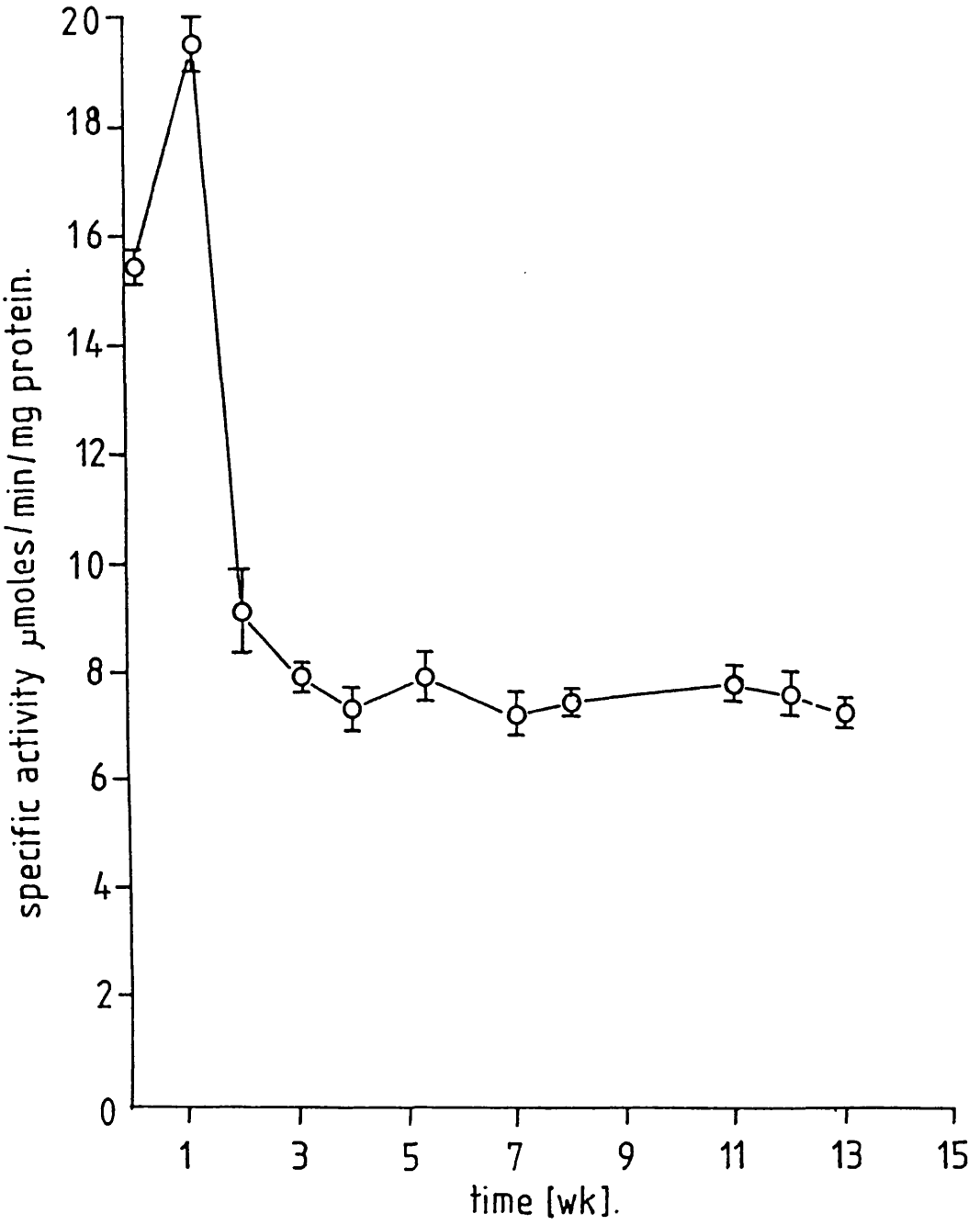


Fig.3 Changes in specific activity of malate dehydrogenase in stored tomato fruit.

Changes in specific activity of malate dehydrogenase in tomato fruit cv. Sonatine, held at 12°C in 6% CO_2, 6% O_2 and 88% N_2, and after standing in ambient atmosphere and temperature. Specific activity during storage (O——O) or after standing in ambient conditions (O---O). Bars are standard errors of the mean of 4 samples.

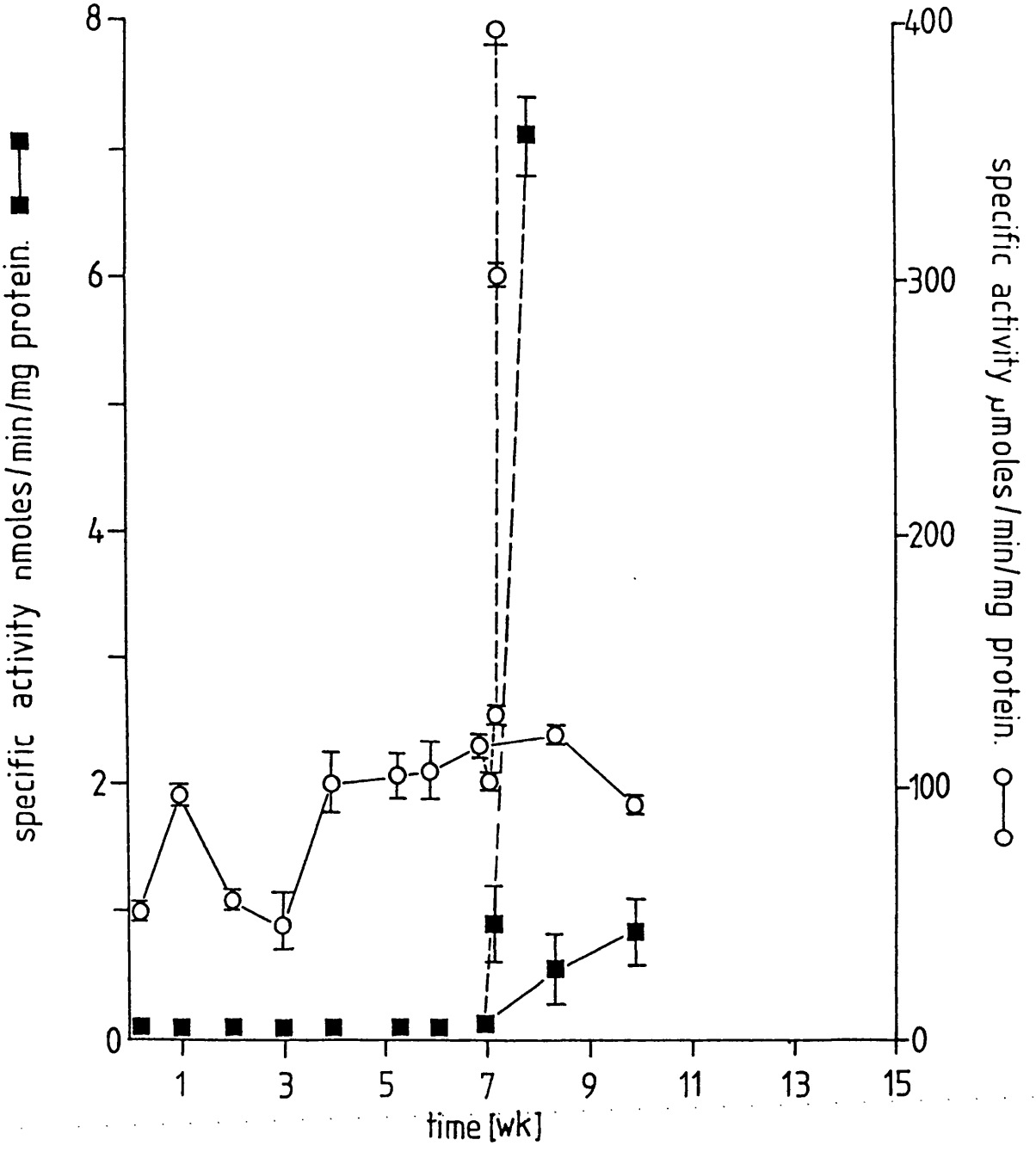


Fig.4 Changes in specific activity of polygalacturonase and acid invertase in stored tomato fruit.

Changes in specific activity of polygalacturonase and acid invertase in tomato fruit cv. Sonatine, held at 12°C in 6% CO_2, 6% O_2 and 88% N_2, and after removal to ambient atmosphere and temperature. Specific activity of polygalacturonase during storage (■—■) or after removal to ambient conditions (■---■). Specific activity of invertase during storage (O——O) or after removal to ambient conditions (O---O). Bars are standard errors of the mean of 4 samples.

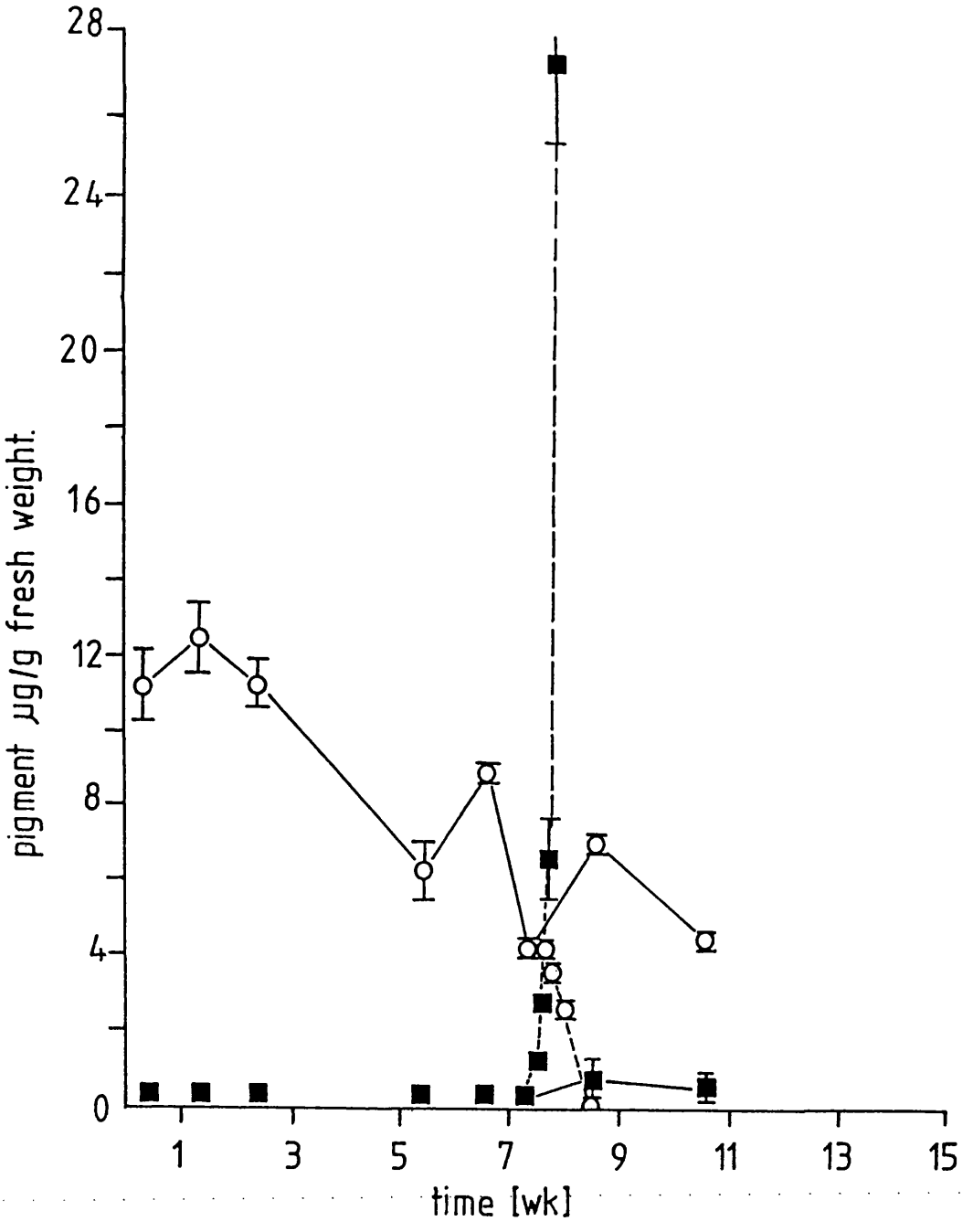


Fig.5 Variation in the concentration of chlorophyll and lycopene in stored tomato fruit.

The concentration of pigments in tomato fruit cv. Sonatine, held at 12°C in 6% CO_2, 6% O_2 and 88% N_2, and after removal to ambient temperature and atmosphere. Concentration of chlorophyll during storage (O——O) or after removal to ambient conditions (O---O). Concentration of lycopene during storage (■——■) or after removal to ambient conditions (■---■). Bars are standard errors of the mean of 6 samples.

6½ weeks that ethylene production became measurable. The concentration then rose to 0.4ppm by week 7 and remained at this level until week 10. When the fruit were removed to a normal atmosphere, there was an immediate increase in the evolution of ethylene to 5ppm which then fell to 3.3ppm (Fig.6).

The concentration of glucose and fructose rose from 407.8mg/g dry wt to reach a peak of 454.1mg/g dry wt after 2 weeks in store. There was a second peak in fructose concentration after 5 weeks but not in glucose. Removal of fruit to a normal atmosphere did not result in an increase in monosaccharide concentration.

Biochemical and Physiological Experiments on Whole Tomato Fruit Stored in the Presence and Absence of Ethylene (2)

The previous experiment demonstrated that in conditions designed to prevent ethylene formation, there was a temporal separation between those aspects of fruit ripening which involve changes in enzyme activities and substrate concentrations of carbohydrate and organic acid metabolism, from those aspects considered to be stimulated by ethylene. Since the previous experiment was performed under conditions in which ethylene synthesis was inhibited, a second experiment was designed to compare the direct effects of ethylene on organic acid metabolism and on enzyme activities known to be stimulated by ethylene. The experimental procedure was identical to the previous experiment, with the exception that fruit were sampled daily. Mature green fruit were stored in glass tanks, one of which was supplemented daily with 27µl/l of ethylene (see Methods, p.25).

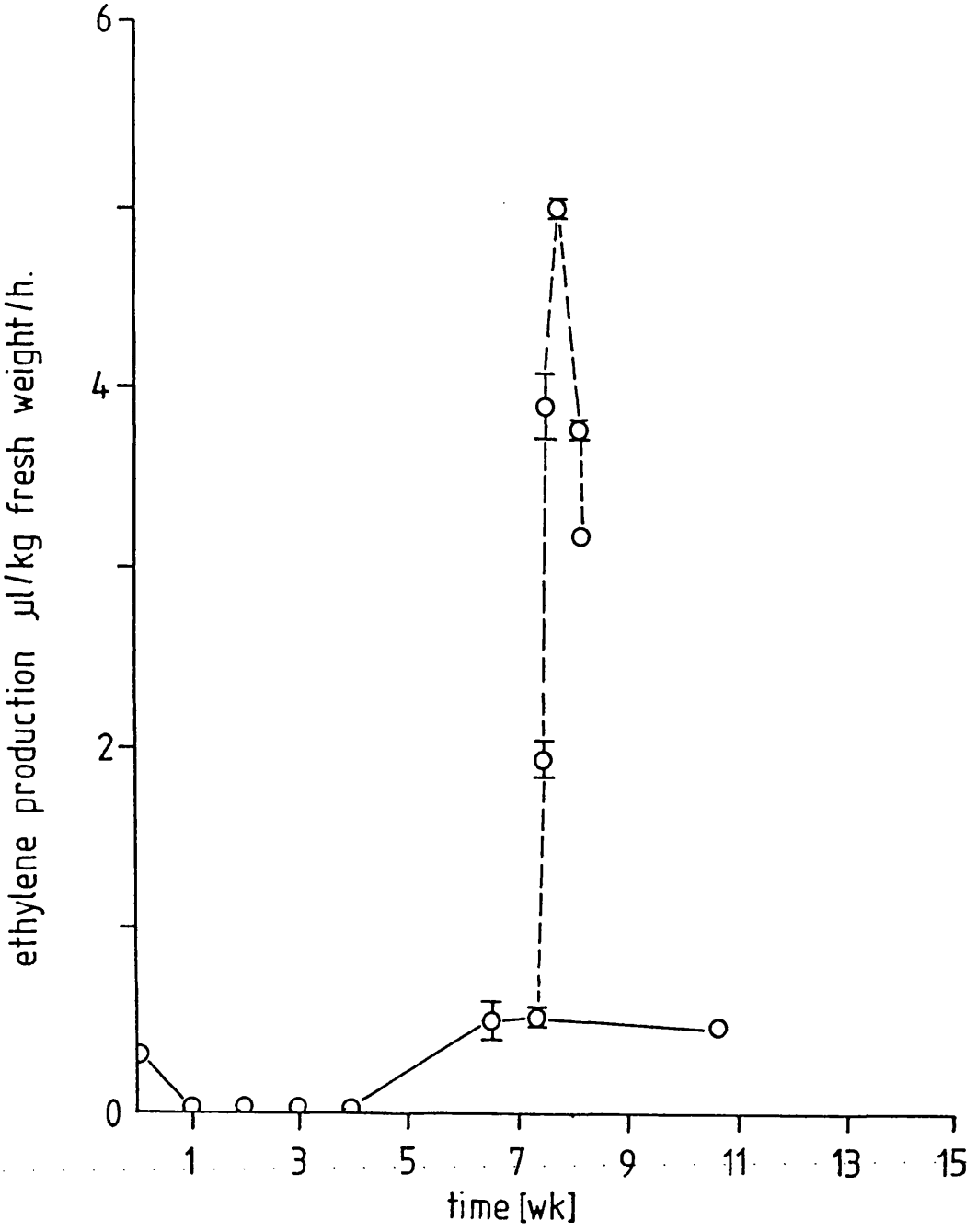


Fig.6 Ethylene evolution from stored tomato fruit.

Ethylene evolution from tomato fruit cv. Sonatine, held at 12°C in 6% CO_2, 6% O_2 and 88% N_2 and after removal to ambient temperature and atmosphere. Ethylene evolution during storage (O——O) or after removal to ambient conditions (O---O). Bars are standard errors of the mean of 6 samples.

Citrate synthase and malate dehydrogenase exhibited similar quantitative changes in both the control and ethylene-supplemented atmosphere to those described in experiment (1). The activity of both enzymes fell rapidly within 84h to 40% of their starting values and remained constant for the duration of the experiment. Citrate synthase activity in samples taken from the control atmosphere fell significantly faster in the first 24h, while those from the ethylene-supplemented atmosphere fell significantly faster between 48 and 72h (Fig.7). The results are expressed as percentage of initial activity rather than specific activity because, during the latter part of the experiment from day 5 onwards, pectin interfered with the Bradford protein assay. NADP-dependent malic enzyme showed no significant change in normal or ethylene-supplemented atmospheres. Malate dehydrogenase activity in the control fruit fell significantly faster in the first 24h; thereafter, there was little difference in the rate of fall for either treatment (Fig.8).

The concentration of malic acid fell immediately the fruit were placed in the tanks and there was no significant difference between the treatments. Malic acid concentration fell to 50% of the starting value in both cases. Citric acid concentration rose slightly in both cases but was not affected by ethylene treatment.

The specific activities of both acid invertase and polygalacturonase started to increase after 5 days in fruit sampled from the control atmosphere, whereas in fruit from the atmosphere supplemented with ethylene, acid invertase began to increase in

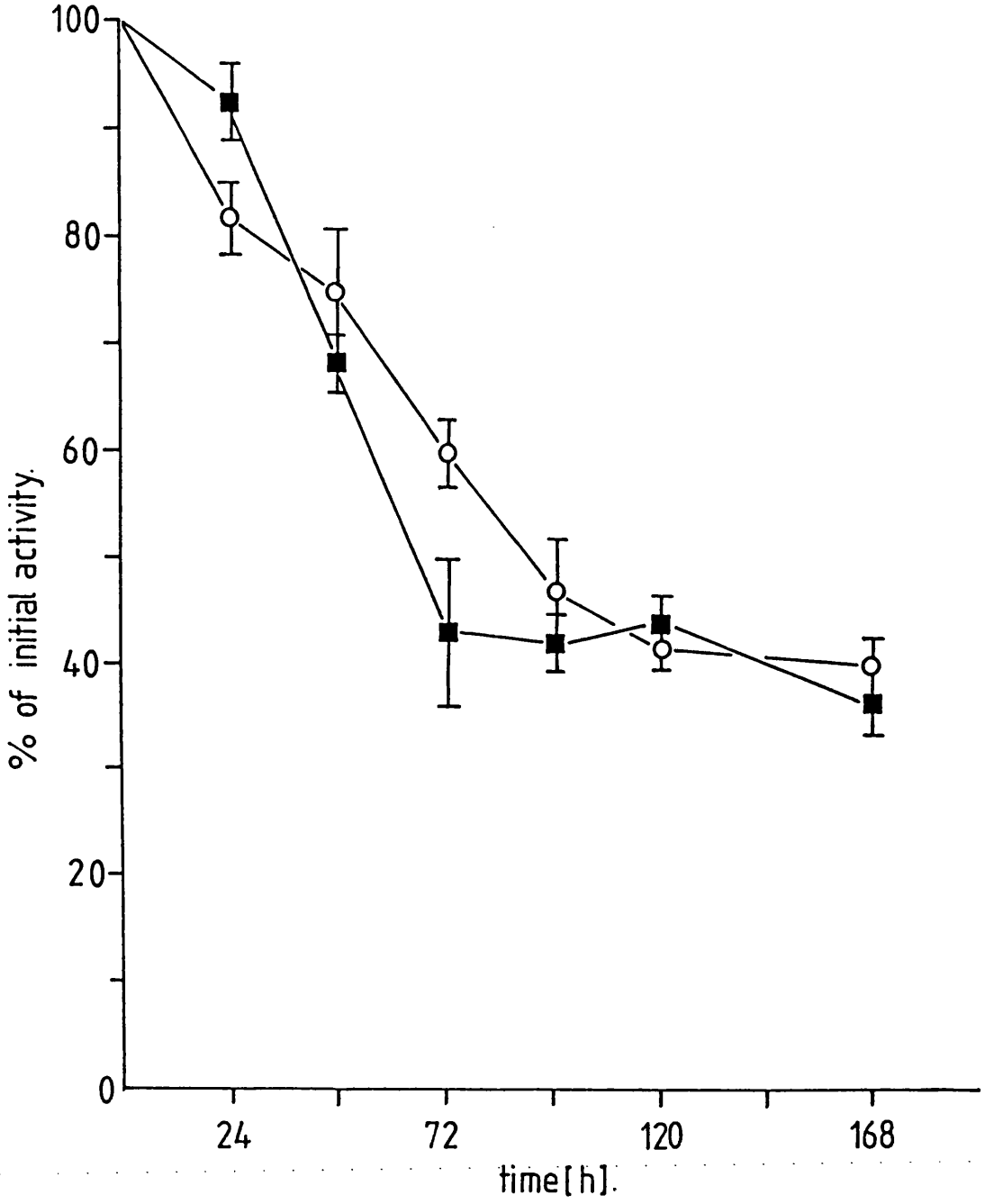


Fig.7 Changes in the activity of citrate synthase in tomato fruit held in the presence or absence of ethylene.

Changes in activity of citrate synthase during ageing of tomato fruit cv. Sonatine, at ambient atmosphere and in an atmosphere containing 27 µl/l of ethylene, both at 22°C. (O——O) ambient atmosphere. (■——■) atmosphere containing 27 µl/l of ethylene. Bars are standard errors of the mean of 4 samples.

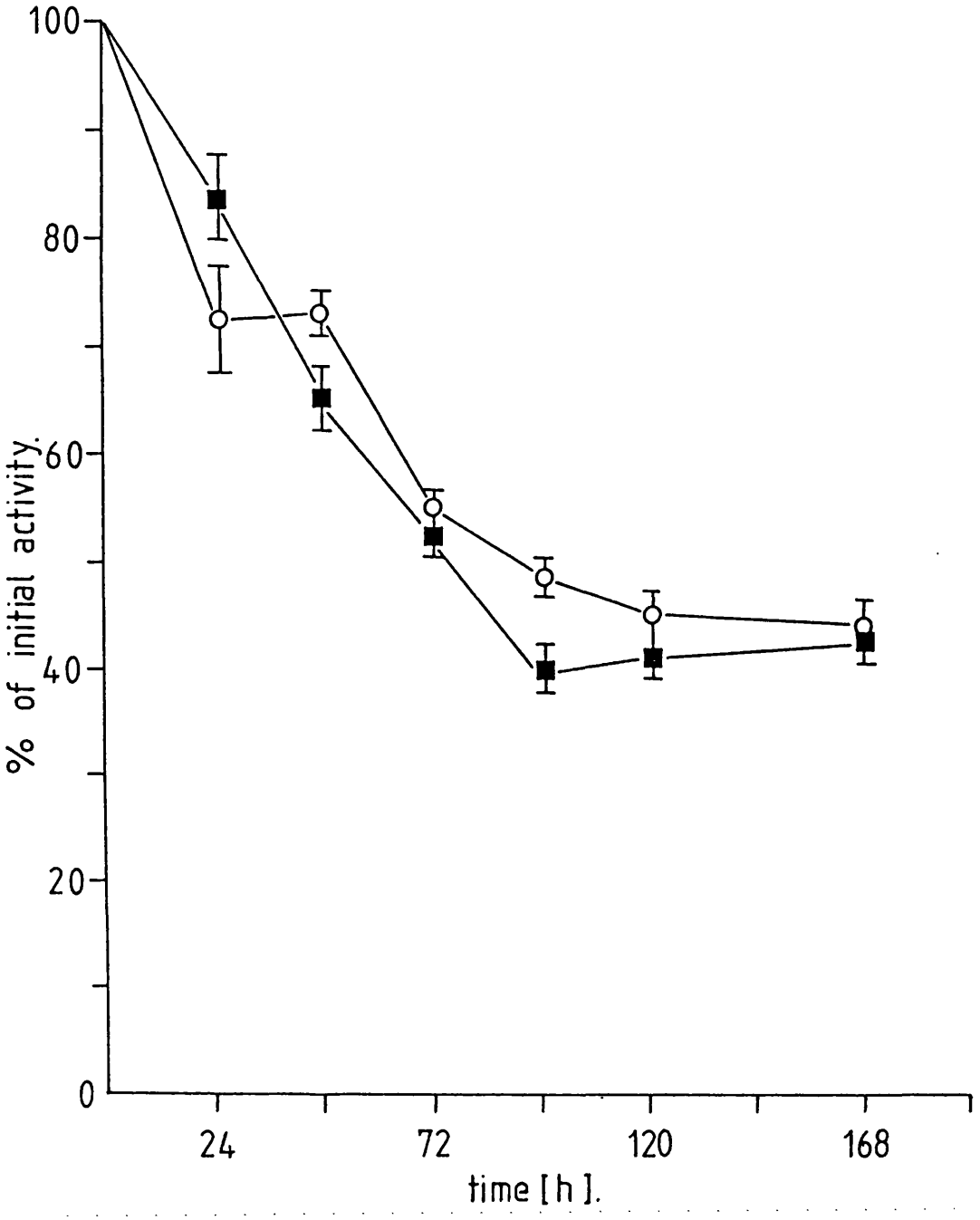


Fig.8 Changes in activity of malate dehydrogenase in tomato fruit held in the presence or absence of ethylene.

Changes in activity of malate dehydrogenase during ageing of tomato fruit cv. Sonatine, at ambient atmosphere and in an atmosphere containing 27 µl/l of ethylene, both at 22°C. (O——O) ambient atmosphere. (■——■) atmosphere containing 27 µl/l ethylene. Bars are standard errors of the mean of 4 samples.

specific activity after 3 days and reached a maximum value by day 5. Polygalacturonase started to increase in specific activity on day 4, and reached a maximum value on day 7 (Fig.9).

Chlorophyll concentration fell to zero by day 6 in fruit taken from the ethylene-supplemented atmosphere, whereas in the control atmosphere over the same time period, the fall in concentration from a similar starting value was 35%. In fruit from the ethylene-supplemented atmosphere, lycopene concentration began to increase between days 3 and 4, and by day 6 had reached 15µg/g fr wt, whereas in fruit from the control atmosphere, lycopene started to appear by day 5 and by day 6 had only reached 4µg/g fr wt (Fig.10).

Biochemical Changes Associated with Ripening in the rin Tomato Mutant

In contrast to Sonatine, the rin mutant exhibited altered profiles for citrate synthase and malate dehydrogenase activities during maturation and ripening. The largest discrepancy was the continued rise in specific activity of citrate synthase from the mature to ripe state, and the 25% fall in citrate concentration during the same period (Fig.11). In addition, the concentration of citric acid at maturation was closer to that of malic acid in the rin compared with Sonatine, where citric acid predominated. The fall in malic acid concentration in the rin was half that found in Sonatine and malate dehydrogenase activity in the rin only fell by 10% between the mature and ripe states (Fig.12). Two determinations were made of NADP-linked malic enzyme activity in the rin. The results were identical and showed no change in specific activity between the

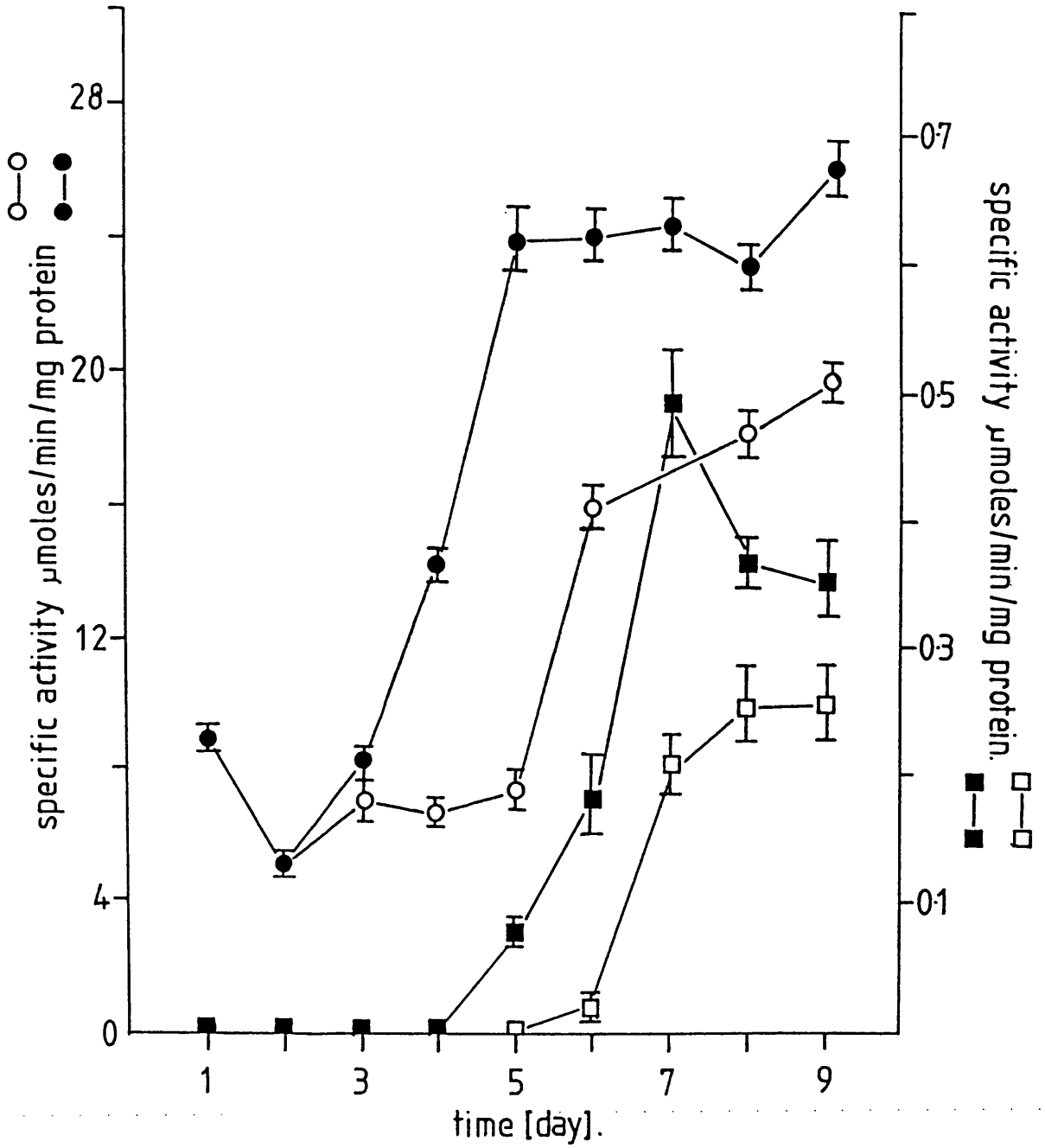


Fig.9 Changes in the specific activity of polygalacturonase and acid invertase in tomato fruit held in the presence or absence of ethylene.

Changes in specific activity of polygalacturonase and acid invertase during ageing of tomato fruit cv. Sonatine, at ambient atmosphere or in an ambient atmosphere containing 27 µl/l of ethylene, both at 22°C. Open symbols denote ambient atmosphere and closed symbols denote atmosphere containing 27 µl/l of ethylene. (□ or ■) polygalacturonase (○ or ●) Invertase. Bars are standard errors of the mean of 4 samples.

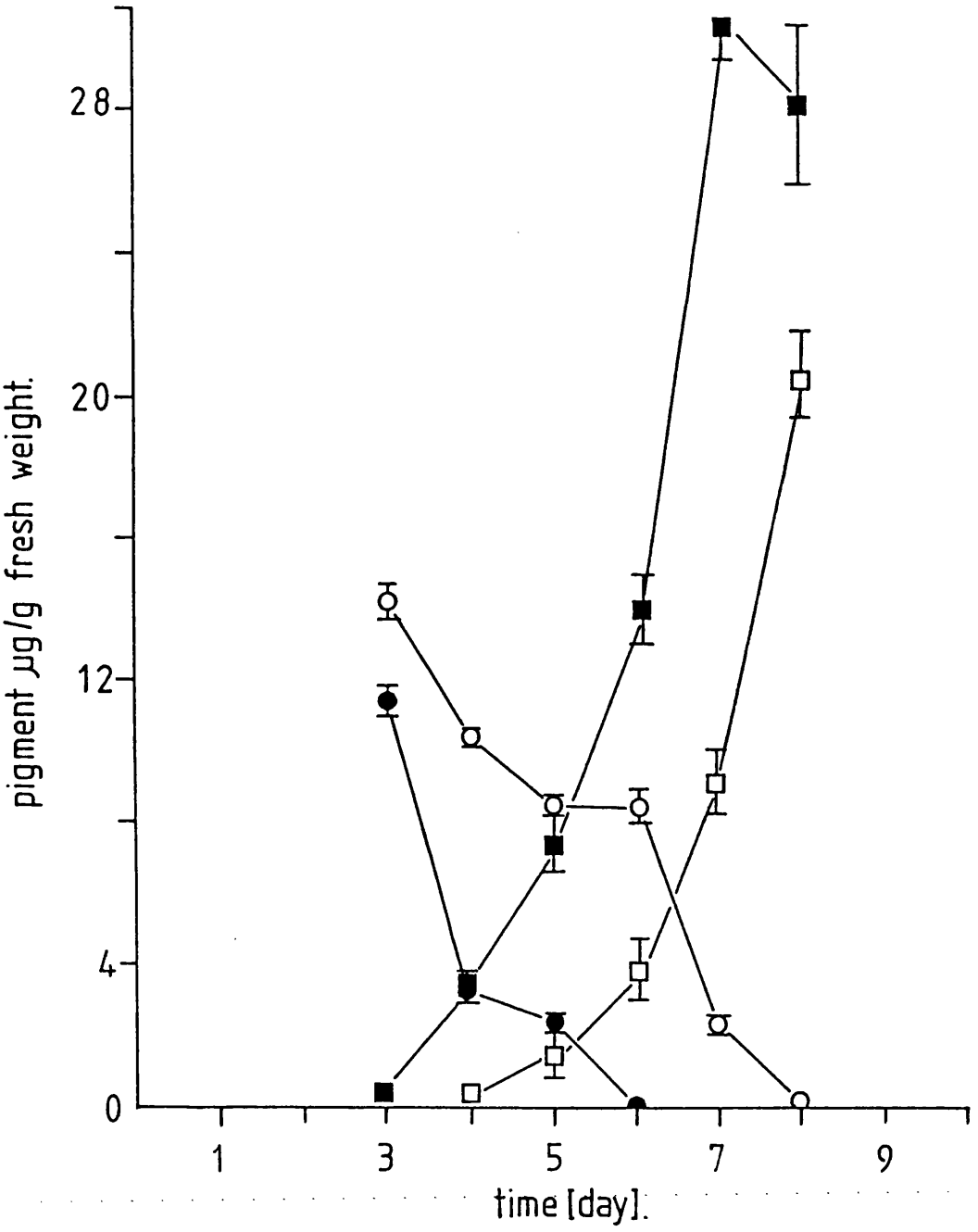


Fig.10 Changes in the concentration of chlorophyll and lycopene in tomato fruit held in the presence or absence of ethylene.

The concentration of chlorophyll and lycopene during ageing of tomato fruit cv. Sonatine at ambient atmosphere or in an ambient atmosphere containing 27 µl/l of ethylene, both at 22°C. Open symbols denote ambient atmosphere and closed symbols denote atmosphere containing 27 µl/l of ethylene. (○ or ●) Chlorophyll (□ or ■) Lycopene. Bars are standard errors of the mean of 6 samples.

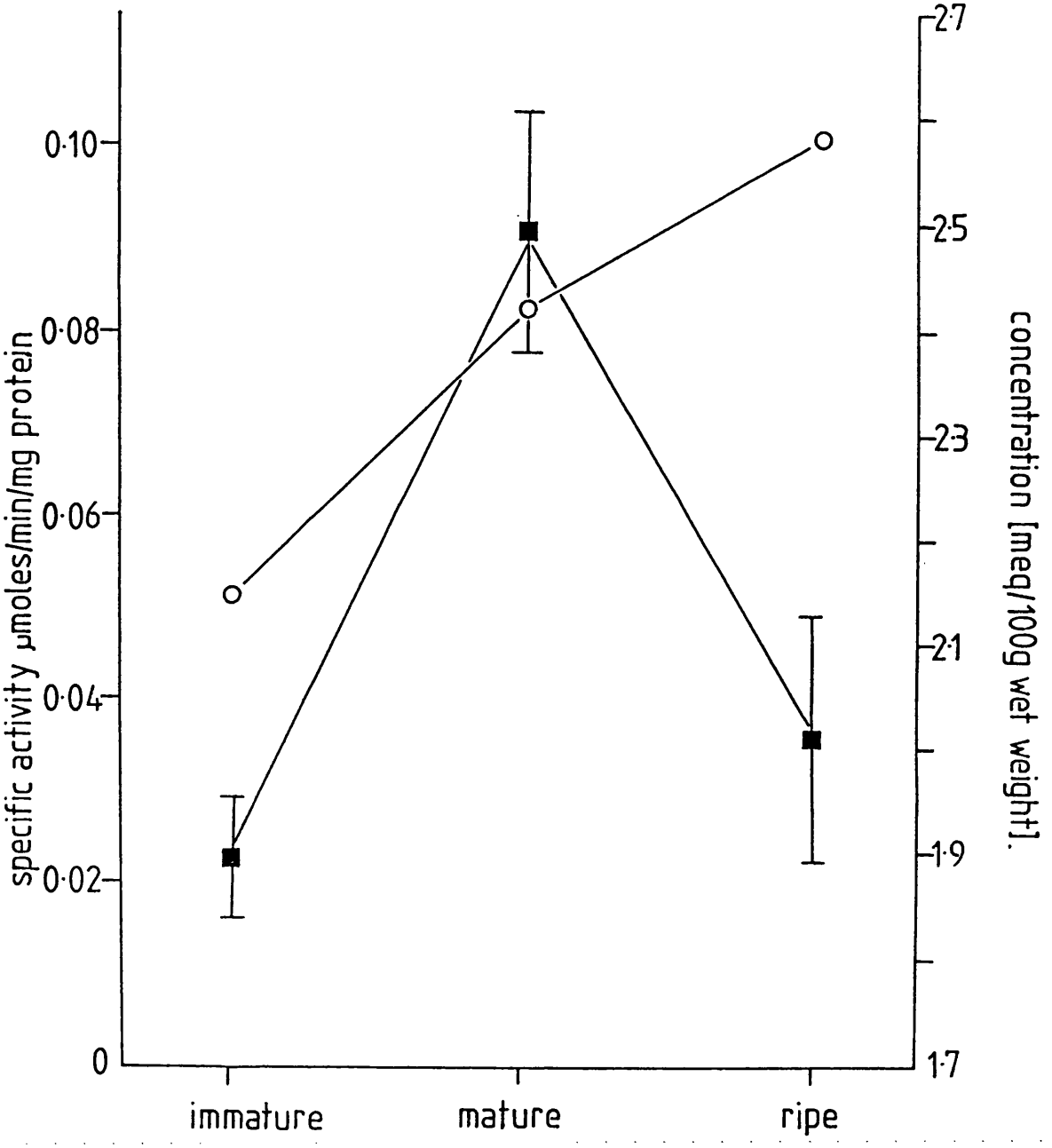


Fig.11 Changes in specific activity of citrate synthase and citrate concentration in rin tomato fruit.

Changes in specific activity of citrate synthase and citrate concentration in rin tomato fruit at different stages of maturation. (O——O) citrate synthase and (■——■) citrate concentration. Bars are standard errors of the mean of 3 samples.

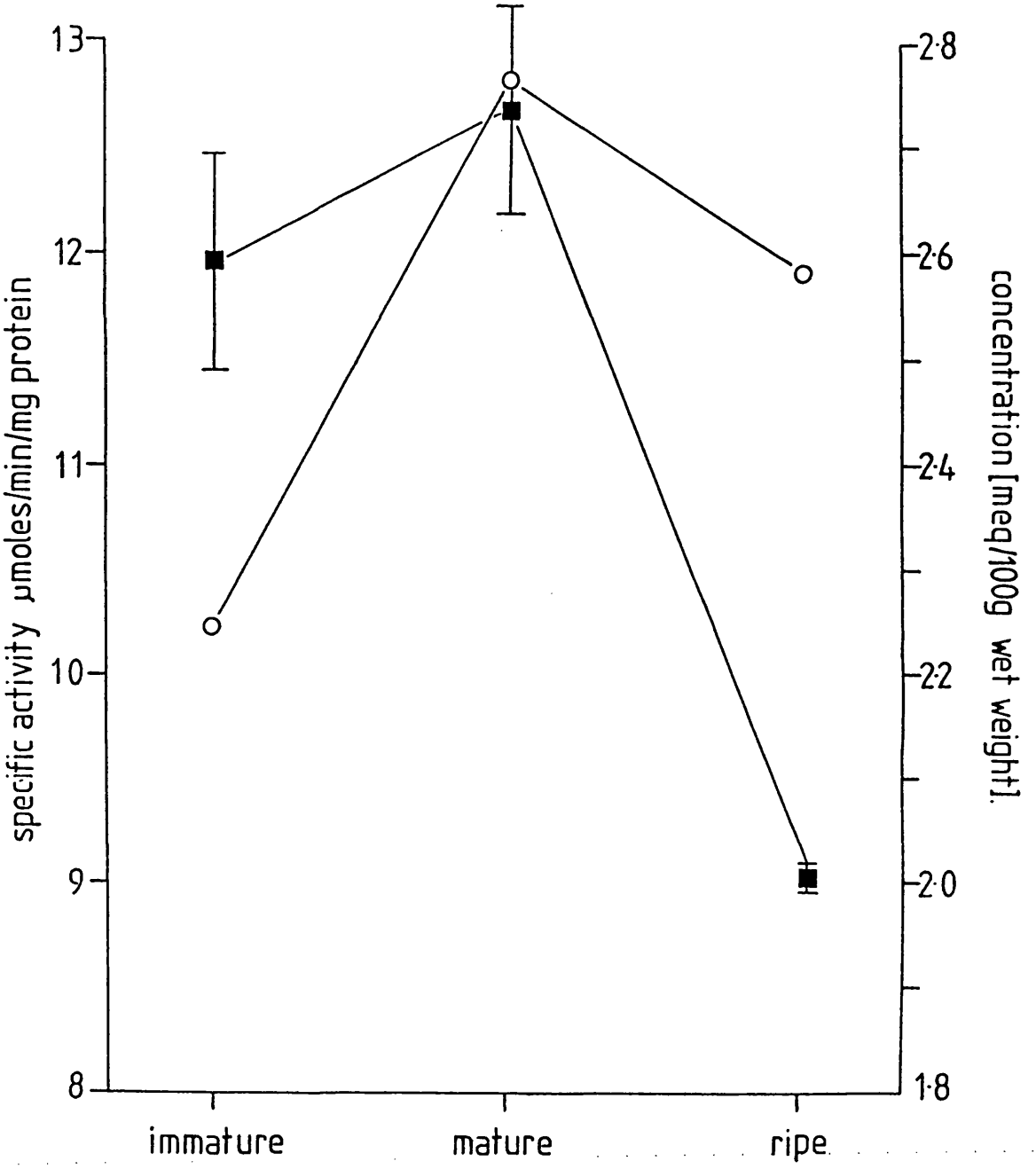


Fig.12 Changes in specific activity of malate dehydrogenase and malate concentration in rin tomatc fruit.

Change in specific activity of malate dehydrogenase and malate concentration in rin tomatc fruit at different stages of maturation. (O——O) malate dehydrogenase (■—■) malate concentration. Bars are standard errors of the mean of 3 samples.

mature and ripe states, but a 40% increase between the immature and mature. In Sonatine, the fall in specific activity of both citrate synthase and malate dehydrogenase to 40-50% of their starting value and the subsequent constancy of activity, suggested a major switch in central metabolism accompanying the change from maturity to ripeness. Accordingly, it was decided to purify and characterise citrate synthase from L. esculentum, to determine if this enzyme possessed any regulatory properties that might interact with the pronounced change in specific activity.

Purification of Citrate Synthase

Initial attempts to purify citrate synthase from L. esculentum utilised DEAE-Sephacel as an anion exchanger. However, although the enzyme bound to this material at pH 8.0, no activity could be recovered after elution with a 0-500mM KCl gradient and this method was abandoned after two attempts ended in failure.

A method using Amicon dye matrex gels was attempted, and Table 2 summarises the binding efficiency of the different gels for citrate synthase from L. esculentum. The results from the binding study indicated that gel Red A was the most favourable matrex with which to purify citrate synthase. This particular gel had been used successfully to purify citrate synthase from Bacillus megaterium and the enzyme had been shown to possess catalytic and regulatory properties which resembled those of eukaryotic citrate synthase (Robinson et al., 1983).

A total of forty units (20 units per column) of citrate

Table 2

The Binding Efficiency of Amicon Matrex Gels for Citrate Synthase from Lycopersicon esculentum

	Column 1.	Column 2.
Green A	35	40
Blue A	80	27
Blue B	7	11
Red A	85	15
Orange A	40	4

Column 1. denotes the percentage of activity loaded onto the column that bound to the gel and was eluted using the conditions below.

Column 2. denotes the percentage of protein loaded onto the gel and eluted as below.

Elution conditions were: 0.5M KCl in 50mM MOPS, pH 7.9. Citrate synthase could not be eluted specifically using 100µM oxaloacetate + 100µM CoA from any of the gels under the specified conditions.

synthase from an 80% ammonium sulphate fraction were loaded onto two small (10 x 0.5cm) columns packed with gel Red A. Both columns had previously been equilibrated with 50mM MOPS, pH 7.8, and the 80% fraction had been dialysed against the same buffer. Citrate synthase bound, and both columns were washed extensively until the A_{280} was below 0.1. The columns were then eluted with the following solutions:

(a) 100μM oxaloacetate + 100μM CoA, pH 7.8

(b) 200μM oxaloacetate + 200μM CoA, pH 7.8

(c) 500μM oxaloacetate + 500μM CoA, pH 7.8

(d) 100μM AcCoA + 100μM citrate, pH 7.8

(e) 100μM oxaloacetate + 100μM CoA + 100μM indole acetic acid, pH 7.8

No eluted activity was detected using any of the above; finally, both columns were eluted with 0.5M KCl and 50% of the activity applied was recovered.

ATP - Sepharose

An 80% ammonium sulphate fraction (15ml, 150 units) were dialysed against the running buffer (50mM MOPS, pH 7.8) and loaded onto the column (1 x 10cm). Fractions were assayed and 146 units of citrate synthase were recovered, indicating that citrate synthase had not bound. Citrate synthase (270 units) from pigeon breast muscle were then loaded and, after washing, 149 units were found to have passed through the column. The column was then eluted with 100μM oxaloacetate + 100μM CoA in 50mM MOPS, pH 7.8 and 95 units of activity were recovered.

Citrate synthase from L. esculentum was finally purified using ammonium sulphate fractionation (50-80% fraction), a linear salt gradient from gel Red A and molecular exclusion chromatography on Sephacryl S-200. The purification is summarised in Table 3 and the elution profiles from gel Red A and Sephacryl are shown in (Figs.13 and 14).

Citrate Synthase - Estimation of M_r by Gel Filtration

The theoretical aspects of M_r determination by gel filtration have been discussed by Andrews (1964). Briefly, the relationship between the approximate M_r of proteins and their elution volumes from gel filtration columns is described by the following equation:

$$Ve = Vo + KdVi$$

where Ve is the elution volume, Vo is the void volume (the elution volume of a solute completely excluded from the internal cavities of the gel), Vi is the volume of solvent imbibed by the gel and Kd is the volume fraction of solvent imbibed by the gel which is accessible to a solute. Gel filtration does not measure the true molecular weight of a protein but its Stokes' radius, which is defined as the radius of a perfect unhydrated sphere having the same rate of passage through the gel as an unknown protein. If unknown and marker proteins are regarded as spherical globular proteins, then in most cases a reasonable estimation of their M_r can be made. In practice, the M_r of an unknown protein can be estimated by plotting $\log_{10} M_r$ of marker proteins against their elution volumes. The relationship is linear over a large M_r range. The elution volume of an unknown protein is then read off against $\log_{10}$ of its M_r.

TABLE 3

	PROTEIN			ENZYME				
STEP	VOLUME OF FRACTION (ml)	CONCENTRATION (mg/ml)	TOTAL (mg)	CONCENTRATION (units/ml)	SPECIFIC ACTIVITY (units/mg protein)	TOTAL (units)	YIELD (%)	PURIFICATION FACTOR
CRUDE EXTRACT	3820	0.55	2101	0.26	0.47	993	100	1
AMMONIUM SULPHATE FRACTION (50 - 80%)	96	5.6	537	5.8	1.03	556	56	2.20
MATREX GEL RED$_A$	24	1.0	24	12	12	288	30	25.5
SEPHACRYL S--200	4.8	0.39	1.87	42.2	108	202	20.3	234.0

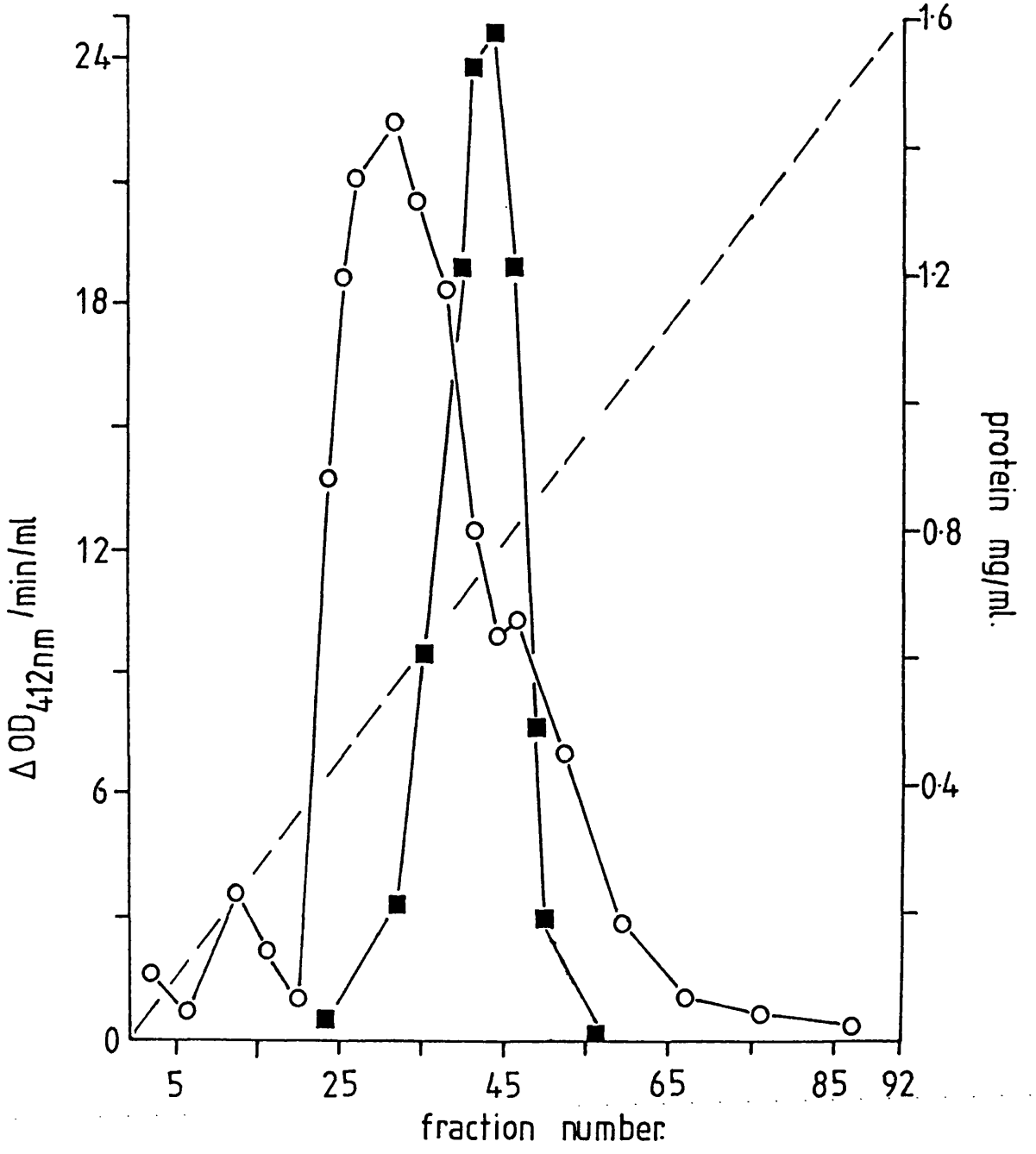


Fig.13 Elution profile of citrate synthase from Matrex Gel Red A.

Elution profile of citrate synthase from Matrex Gel Red A. The column was equilibrated with 50mM MOPS containing 50mM KCl, pH 7.8. The column was eluted with a linear KCl gradient between 50-500mM in 50mM MOPS, pH 7.8, at a flow rate of 15ml/h. The open circles represent protein, the closed squares citrate synthase activity and the dashed line the salt gradient.

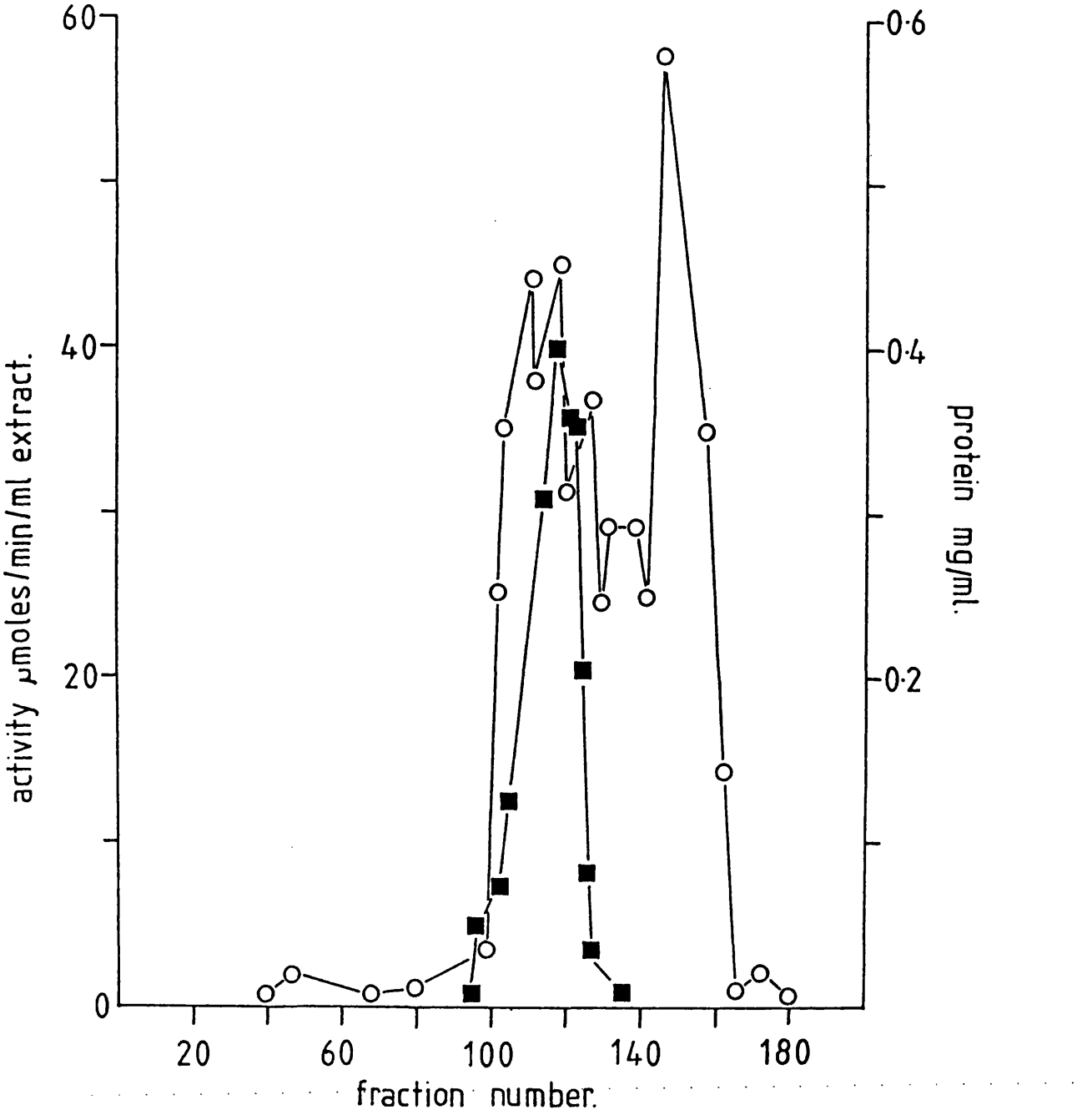


Fig.14 Elution profile of citrate synthase from Sephacryl S-200.

Elution profile of citrate synthase from Sephacryl S-200. The column was equilibrated with 50mM MOPS, pH 7.8. The enzyme was loaded in a total volume of 1.5ml and the column was eluted at a flow rate of 5.0ml/h. The open circles represent eluted protein and the closed squares citrate synthase activity.

This relationship holds for most globular proteins, although some proteins tend to dissociate into sub-units under certain conditions of pH and ionic concentration. Examples are bovine haemoglobin and lactoglobulin.

The following proteins of known molecular weight were used as markers: ferritin (450,000), catalase (240,000), aldolase (158,000), bovine serum albumin (68,000), chymotrypsinogen (25,000) and cyctochrome C (12,500). One ml of buffer solution containing 1mg of each of the above proteins was applied to a (1 x 100cm) column. Proteins were detected in the column effluent by the following methods. Cytochrome C and ferritin are coloured proteins and were detected visually: the remaining fractions were measured at 280nm to detect catalase, aldolase, and bovine serum albumin. Citrate synthase was detected by assaying its activity. The column elution profile (Fig.15) shows well defined peaks with no overlap. The void volume was 59ml, well inside the elution volume of the largest protein, ferritin (65ml). The plot of elution volume against $\log_{10} M_r$ (Fig.16) indicates a good linear relationship, with the exception of cytochrome C. In subsequent experiments, ribonuclease A(13,600) was substituted for cytochrome C and found to give a better fit. The estimated M_r of citrate synthase from L. esculentum is 104,000. This compares with an M_r of 100,000 for citrate synthase from bean, cauliflower, wheat and maize, and 65,000 for mango (Weitzman and Danson, 1976).

Determination of Purity and Sub-Unit M_r

From the active sephacryl fractions, 5µg of protein were loaded onto

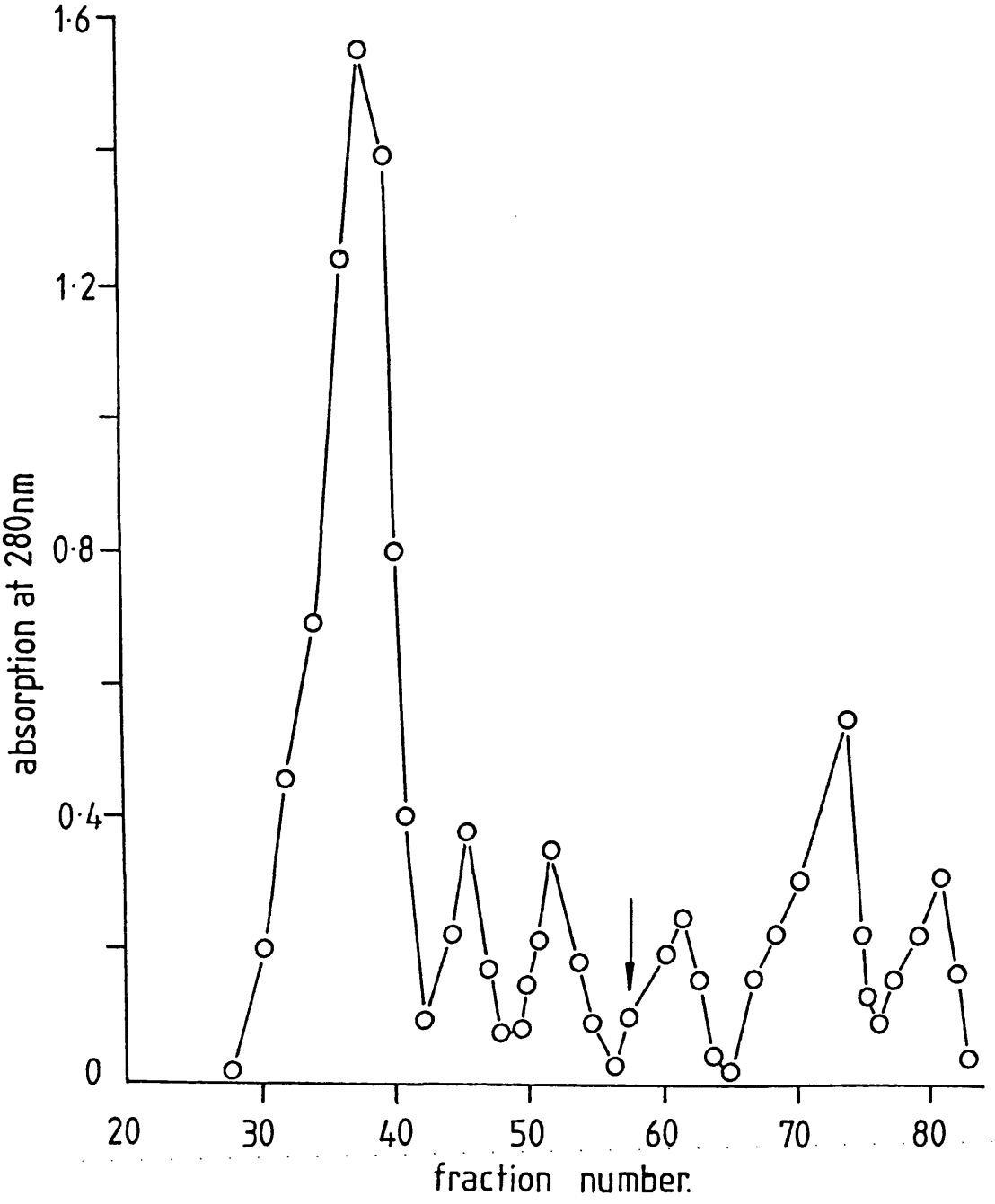


Fig.15 Elution profile of marker proteins from Sephacryl S-200.

Elution profile of marker proteins used to determine the M_r of citrate synthase. From left to right: ferritin, catalase, aldolase, BSA, chymotrypsinogen and cytochrome C. The arrow indicates the position at which purified citrate synthase from L. esculentum was detected. Proteins were loaded simultaneously at a concentration of 1.0mg/ml and the column eluted at a flow rate of 5.0ml/h.

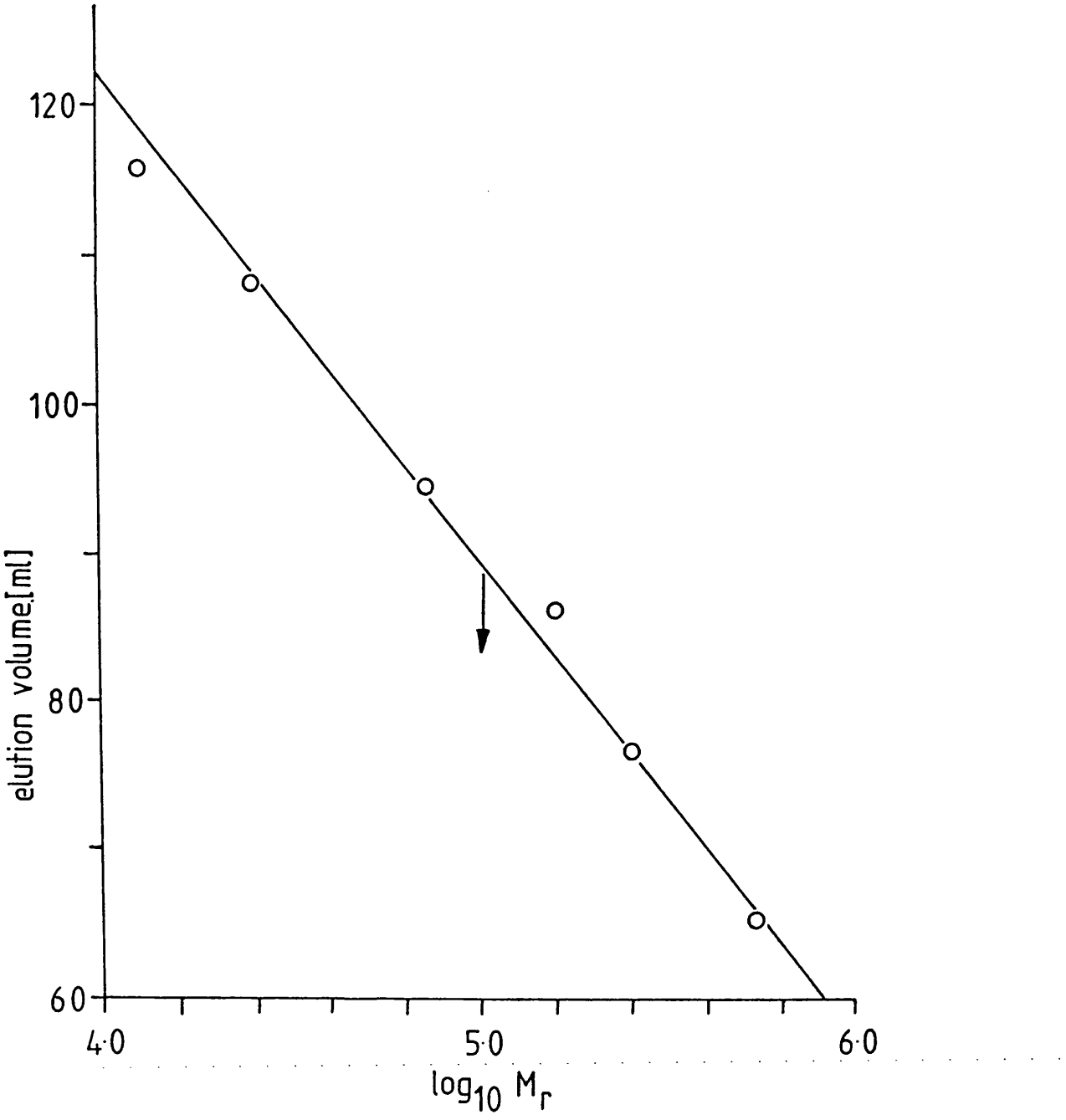


Fig.16 Estimation of citrate synthase M_r.

Plot of elution volume against $\log_{10}M_r$ of Sephacryl S-200 marker proteins. From left to right marker proteins are: cytochrome C (12,500), chymotrypsinogen (25,000), BSA (68,000), aldolase (158,000), catalase (240,000) and ferritin (450,000). The arrow indicates the approximate $\log_{10}M_r$ of citrate synthase from L. esculentum. Results are the mean of 2 determinations.

7.5% SDS polyacrylamide gels. The gels were run at 3mA per gel for approximately 4h. After destaining, the peak fraction was seen to be homogeneous and the majority of the other fractions contained two low molecular weight contaminants. Fig.17 illustrates the scan of the peak tube. To determine the sub-unit M_r the following proteins were used: myoglobin (17,000), carbonic anhydrase (30,000), aldolase (40,000) and E. coli pyruvate dehydrogenase (56,000, 83,000 and 100,000). Eight µg of each marker and 8µg of citrate synthase were loaded and the gels run as above. However, the result was ambiguous as the 56,000 M_r E. coli pyruvate dehydrogenase sub-unit did not separate well from citrate synthase and the best estimate for the M_r of citrate synthase was 50-60,000. A further purification of citrate synthase resulted in two distinct bands separated by 1.5cm. Ten µg of this preparation were run with 5µg each of the following Beohringer markers: trypsin inhibitor (21,500), bovine serum albumin (68,000) and RNA polymerase (α39,000, β155,000 and β'165,000). Fig.18 illustrates the relationship between $\log_{10}$ and migration distance; from this the sub-unit M_r of the two bands were estimated to be 51,000 and 35,000. In an attempt to determine which band corresponded to citrate synthase, 10µg of protein were run on a 5% non-denaturing gel. It was hoped that the two bands would separate enough to allow their excision from the gel and subsequent staining for activity. This was not possible, however, as both proteins repeatedly ran as a smear and did not separate into discrete bands. The non-denaturing gels were run without a separate stacking gel and this undoubtedly contributed to the poor resolution. As will be seen later, when a stacking gel was added the results were greatly improved.

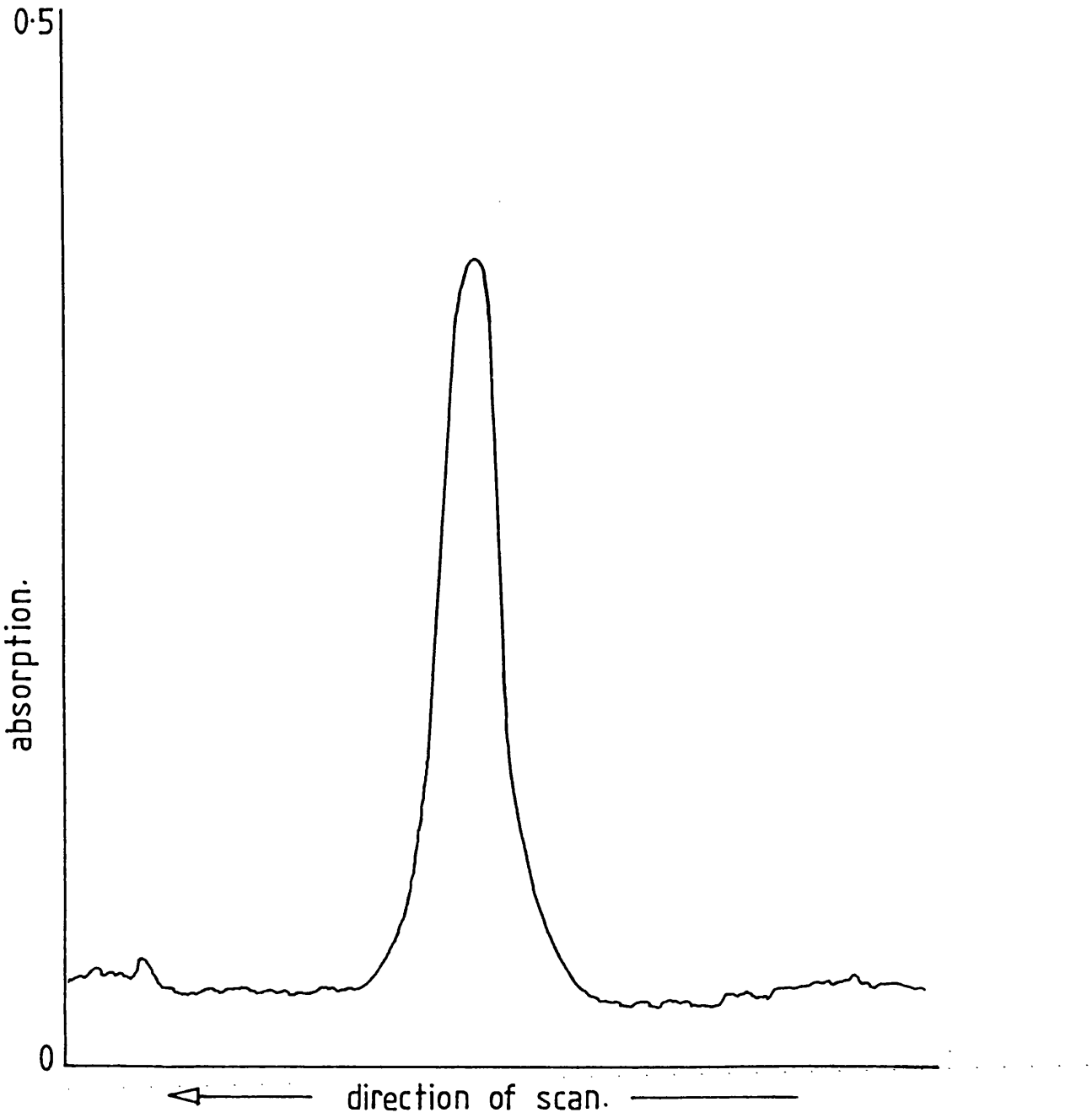


Fig.17 Gel scan of purified citrate synthase.

Spectrophotometric trace of a 7.5% SDS gel showing a single peak representing citrate synthase from L. esculentum. Scan speed was 10sec/cm.

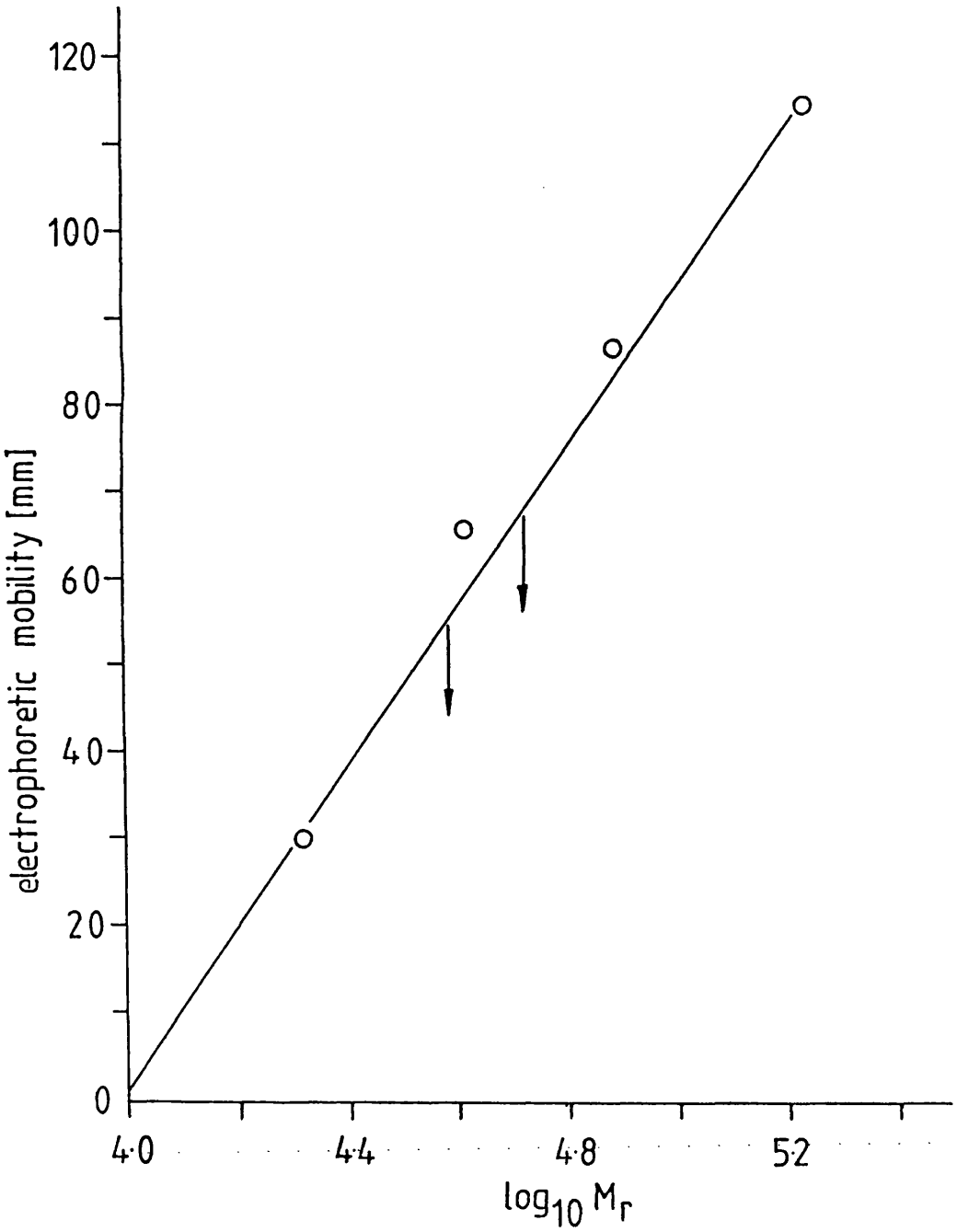


Fig.18 Estimation of citrate synthase sub-unit M_r.

Plot of electrophoretic mobility against $\log_{10} M_r$ of marker proteins used in the determination of the sub-unit size of citrate synthase from L. esculentum. From left to right the protein markers are: trypsin inhibitor (21,500), RNA polymerase α (39,000), BSA (68,000) and the mean of RNA polymerase β (155,000) and RNA polymerase β' (165,000). The arrows indicate the $\log_{10} M_r$ of the 2 unknown bands.

pH Profile of Purified Citrate Synthase

All assays were performed in 50mM MOPS, and the pH limits were 7.0 - 9.0. The pH of the entire reaction mixture was checked before neutralised oxaloacetate was added. Fig.19 shows the broad bell-shaped profile from pH 7.6 - 8.4 with a maximum between pH 7.9 - 8.0. This compares with a pH maximum of 7.5 for citrate synthase from Phaseolus vulgaris in 40mM phosphate buffer (Greenblatt and Sarkissian, 1973) and a maximum of pH 8.5 for citrate synthase from maize scutellum in 20mM glycylglycine buffer (Barbareschi et at., 1974).

Thermal Stability Studies on Purified Citrate Synthase

Part of the rationale for purifying citrate synthase was that thermal stability studies might indicate the presence of one or more isoenzymes. If this were the case, then the loss of a particular isoenzyme could explain the characteristic fall in specific activity that occurs between maturation and ripeness.

Citrate synthase from L. esculentum was incubated in a heated water-bath at the following temperatures: 40°C, 45°C, 47.5°C, 50°C and 55°C. At each temperature, 16µg of enzyme were withdrawn and assayed at 1, 2, 4, 6, 8 and 10min intervals. The results are shown in Fig.20 and, apart from the 55°C plot, all appear monophasic. Concurrently, the effects of the two substrates and one of the products on thermal stability were measured. A temperature of 47.5°C was chosen, since at this temperature 50% inactivation occurs between 3 and 4min. Citrate synthase (200µl) from the peak Sephacryl S-200 fractions was incubated with an equal volume of the

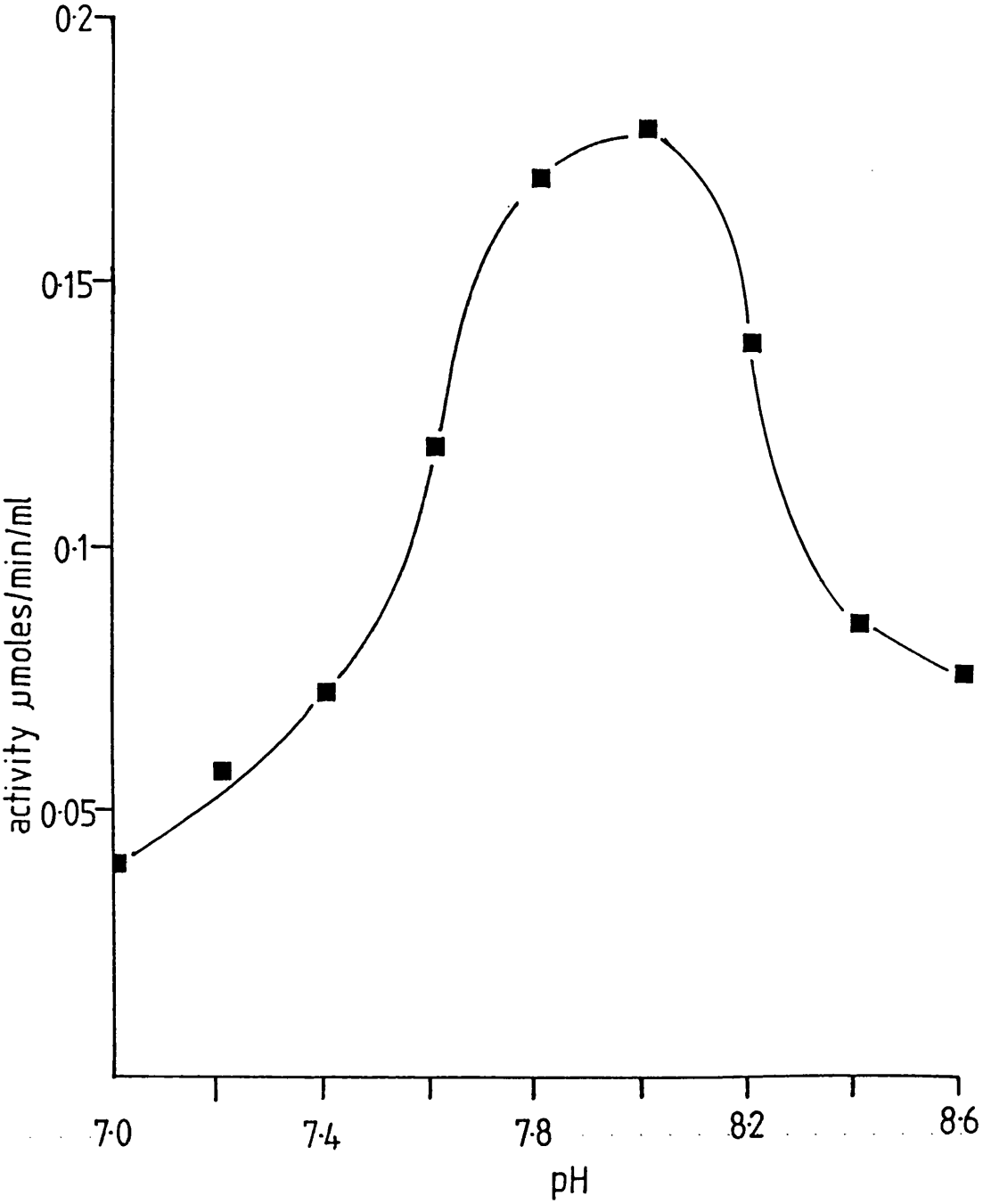


Fig.19 The effect of pH on citrate synthase activity.

The reaction mixture contained 50mM MOPS, 0.2mM acetyl-CoA, 0.1mM DTNB and enzyme. The pH of the reaction mixture was checked before neutralised oxaloacetate (0.2mM) was added to start the reaction. The volume of the reaction mixture was 1ml.

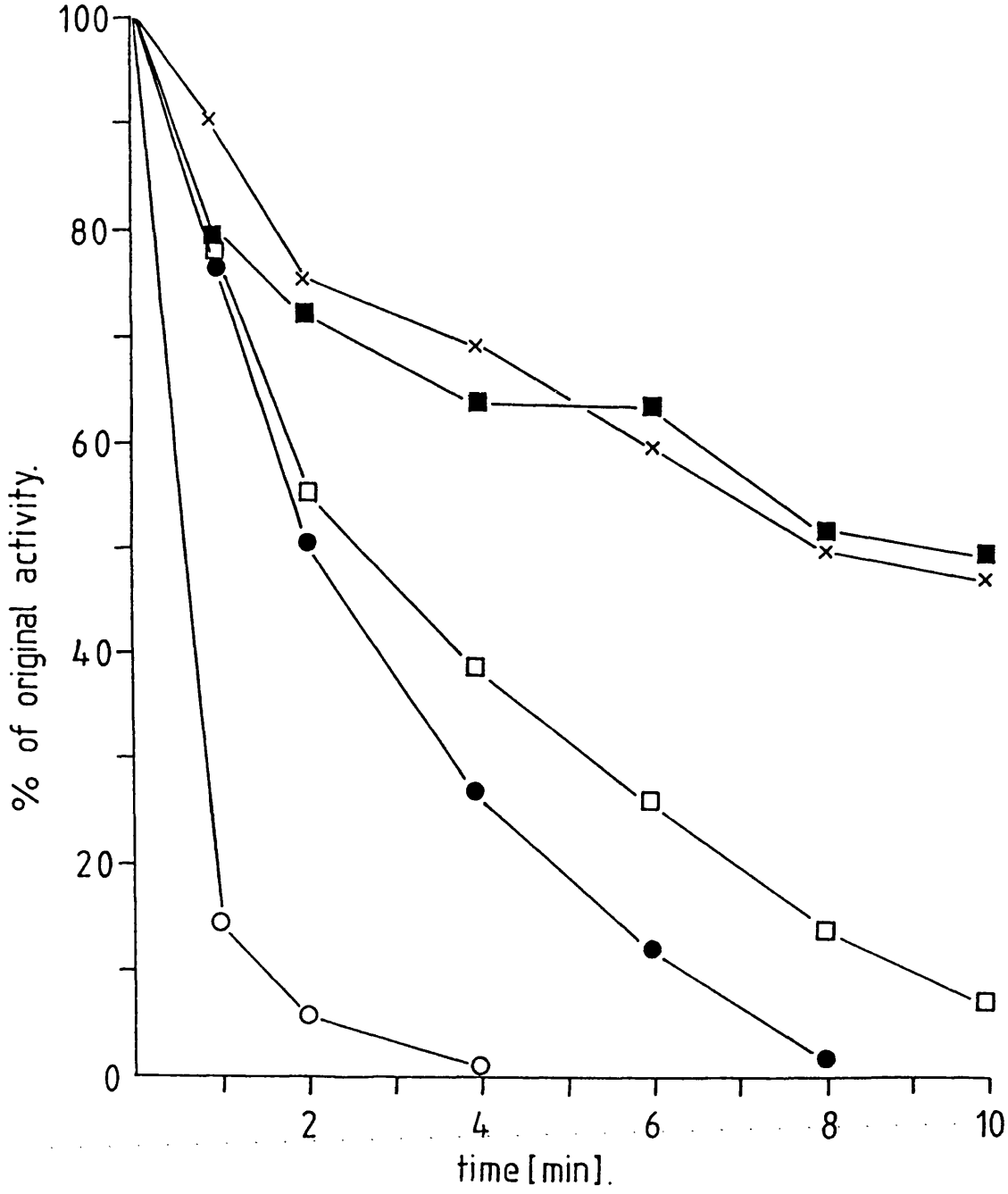


Fig.20 Thermal inactivation of purified citrate synthase.

Inactivation of purified citrate synthase from L. esculentum, incubated at the following temperatures: (○——○, 55°C), (●——●, 50°C), (□—□, 47.5°C), (■—■, 45°C) and (x——x, 40°C).

following: 100mM, 10mM and 1mM citric acid (trisodium salt), 100mM, 10mM and 1mM isocitric acid (trisodium salt), 10mM and 1mM oxaloacetate and 10mM acetyl-CoA. The results are illustrated in Figs.21, 22 and 23. Oxaloacetate affords by far the best protection against thermal inactivation, whereas acetyl-CoA appears actually to enhance the rate of inactivation. Relatively high concentrations of citrate and isocitrate confer limited protection against thermal inactivation, but only for short periods.

Activation of Purified Citrate Synthase by Indole Acetic Acid

Sarkissian (1966, 1970) reported that citrate synthase from bean hypocotyl was activated and modified in molecular size by the plant hormone indole acetic acid (IAA). However, Brock and Fletcher (1969) could find no evidence for activation when they repeated the work. In the present work, the effect of IAA was examined at two concentrations, 4×10^{-5}M and 4×10^{-9}M, at substrate concentrations ranging from 10X Km to 0.5X Km. IAA was allowed to incubate for 3min at 30°C with 20µg of citrate synthase in 50mM MOPS, pH 7.9, before the addition of the remaining assay constituents. Additionally, IAA and citrate synthase were incubated for the same time in the presence of the assay constituents with the exception of oxaloacetate. In no case was any evidence for activation found.

Inhibition of Purified Citrate Synthase by DTNB

One of the characteristics that distinguishes plant citrate synthases from animal citrate synthases, is that many of the former appear to be inactivated to some degree by sulphydryl-blocking agents, such as

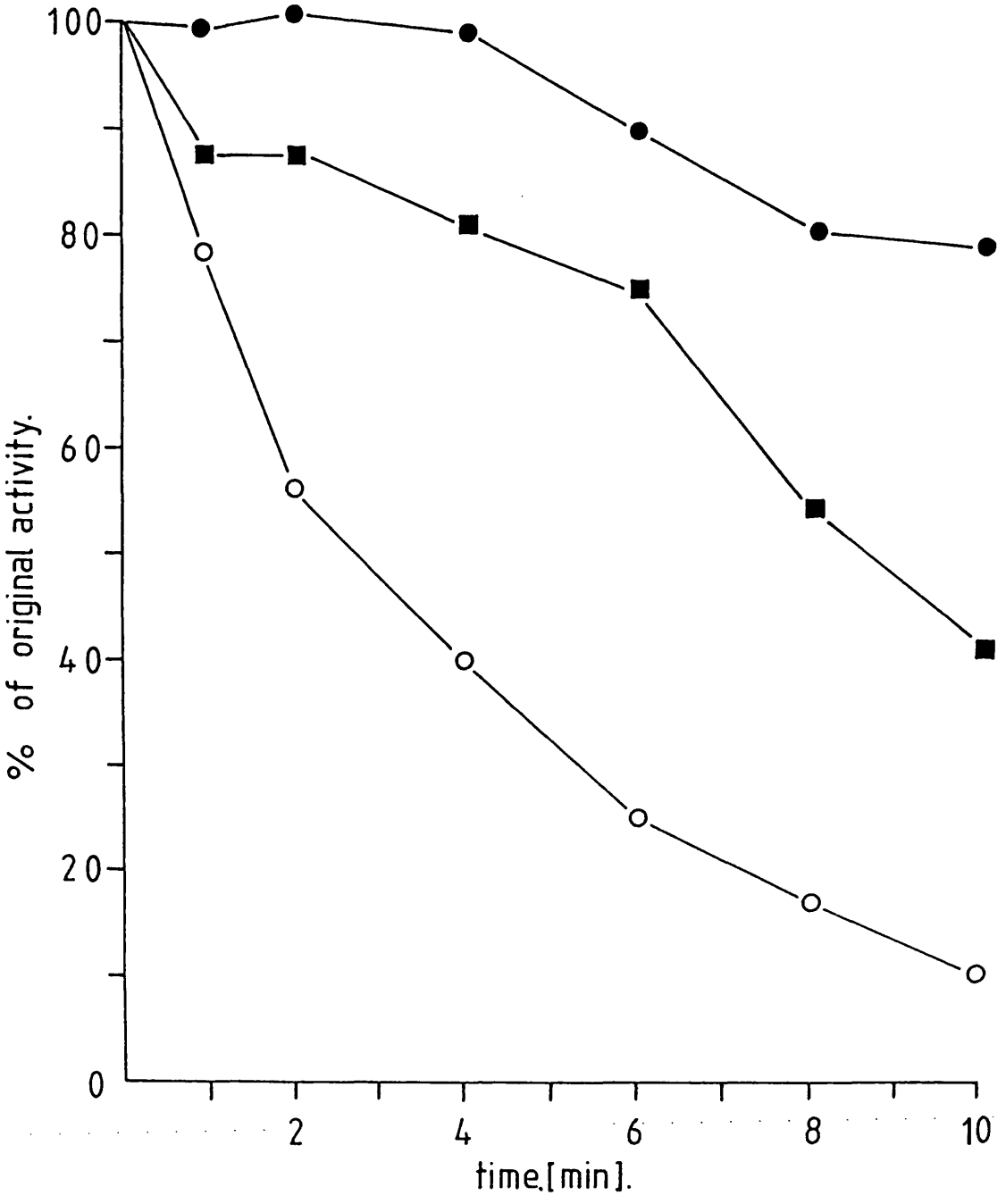


Fig.21 Thermal inactivation of citrate synthase in the presence of citrate.

Inactivation of purified citrate synthase from L. esculentum incubated at 47.5°C in the presence of: (O——O, 1mM citrate), (■——■, 10mM citrate) and (●——●, 100mM citrate).

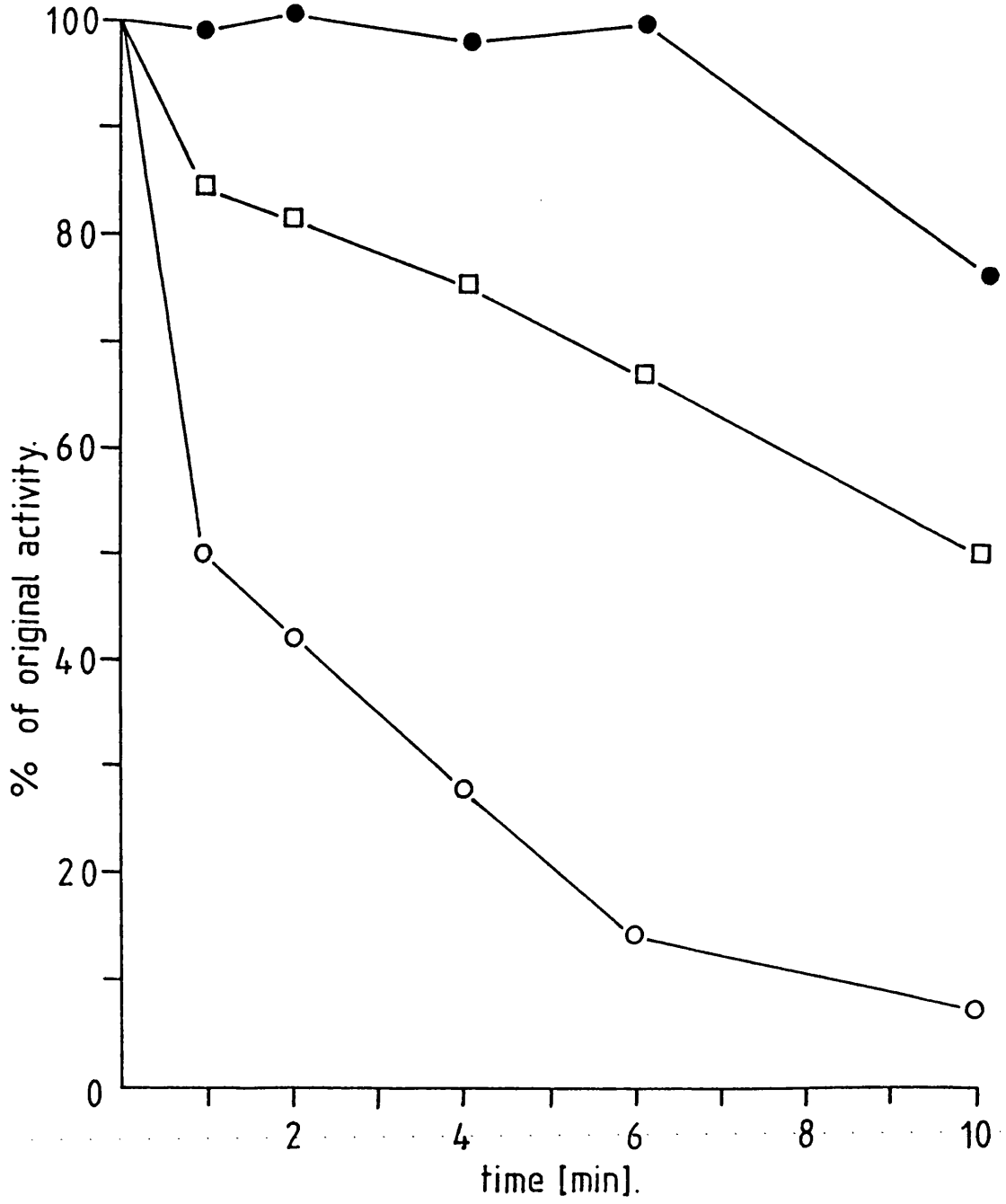


Fig.22 Thermal inactivation of citrate synthase in the presence of isocitrate.

Inactivation of purified citrate synthase from L. esculentum incubated at 47.5°C in the presence of: (O——O, 1mM isocitrate), (□—□, 10mM isocitrate) and (●——●, 100mM isocitrate).

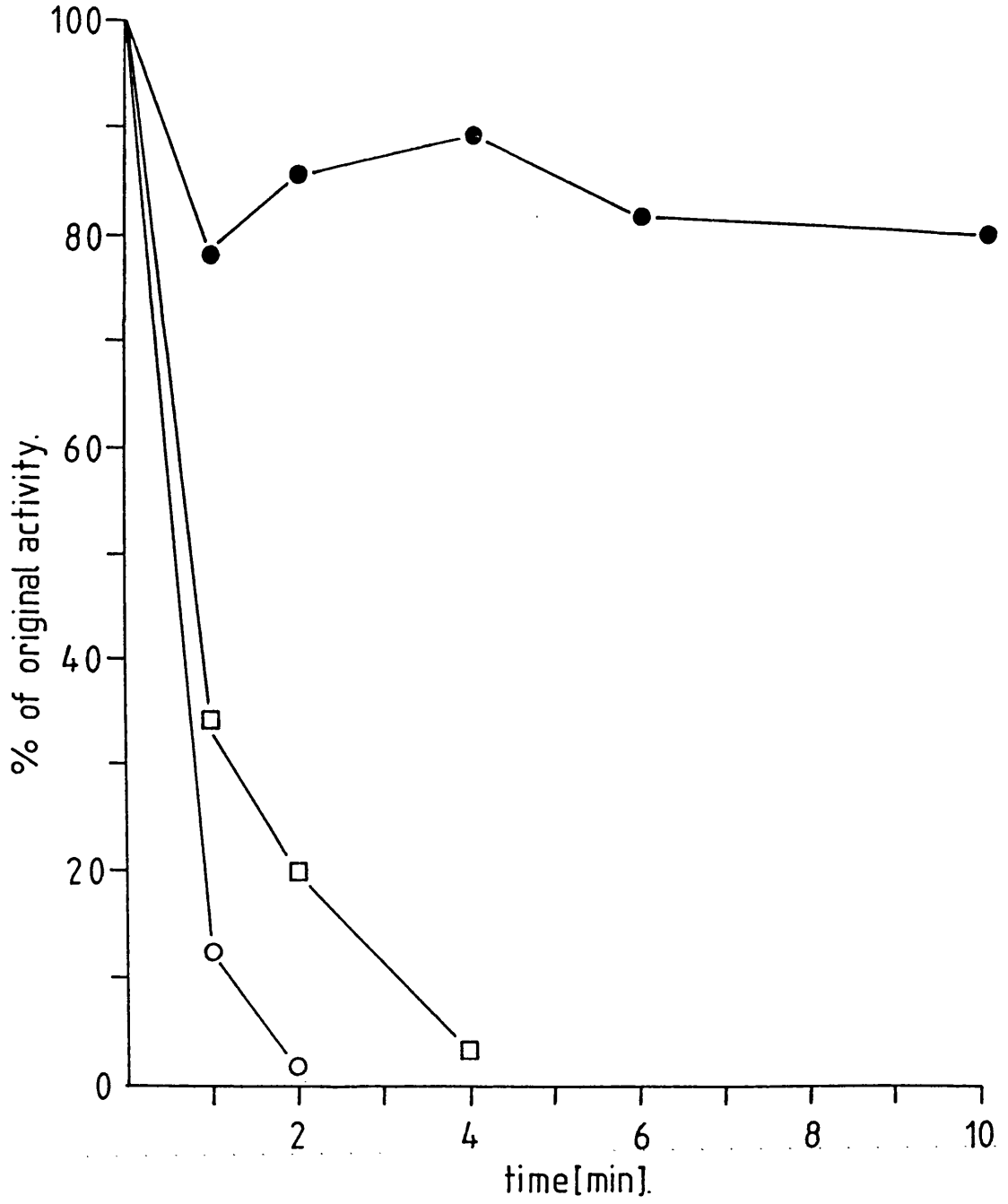


Fig.23 Thermal inactivation of citrate synthase in the presence of acetyl-CoA and oxalacetate.

Inactivation of purified citrate synthase from L. esculentum incubated in the presence of: (O——O, 10mM acetyl-CoA), (□—□, blank containing buffer only) and (●——●, 1mM oxalacetate).

DTNB, p-hydroxy-mercuribenzoate and N-ethyl maleimide (Weitzman and Danson, 1976). The inactivation of citrate synthase from L. esculentum by 5mM DTNB is shown in Fig.24. Protein (92µg) in 250µl of 50mM MOPS, pH 7.9, was incubated with an equal volume of 10mM DTNB at 25°C. At 15min intervals, 80µl of the mixture were added to the normal assay constiuents. An identical amount of pig heart citrate synthase was used in a parallel experiment. In the presence of 5mM DTNB, citrate synthase from L. esculentum showed a slow decline in activity over 30min, then a rapid drop to 5% of the starting activity between 30-45min. The activity of pig heart citrate synthase fell 8% over the same period, and a control consisting of an equal amount of citrate synthase from L. esculentum incubated in 50mM MOPS without DTNB fell 16% in activity.

Kinetic Properties of Purified Citrate Synthase

The Km for each substrate was determined by varying one substrate from 200 - 6.25µM while keeping the other at a saturating concentration (200µM). Care was exercised throughout to ensure that temperature and timing remained as constant as possible. Duplicates of all assays were performed and plots of v against S were hyperbolic. Km values for oxaloacetate and acetyl-CoA were determined to be 19µM and 18µM respectively. These figures compare with 12µM and 28µM respectively for citrate synthase from Phaseolus vulgaris (Greenblatt and Sarkissian, 1973), 34µM and 4µM for mitochondrial citrate synthase from maize scutellum (Barbareschi et al., 1974) and 3.6µM and 2.8µM for rat liver citrate synthase (Moriyama and Srere, 1971).

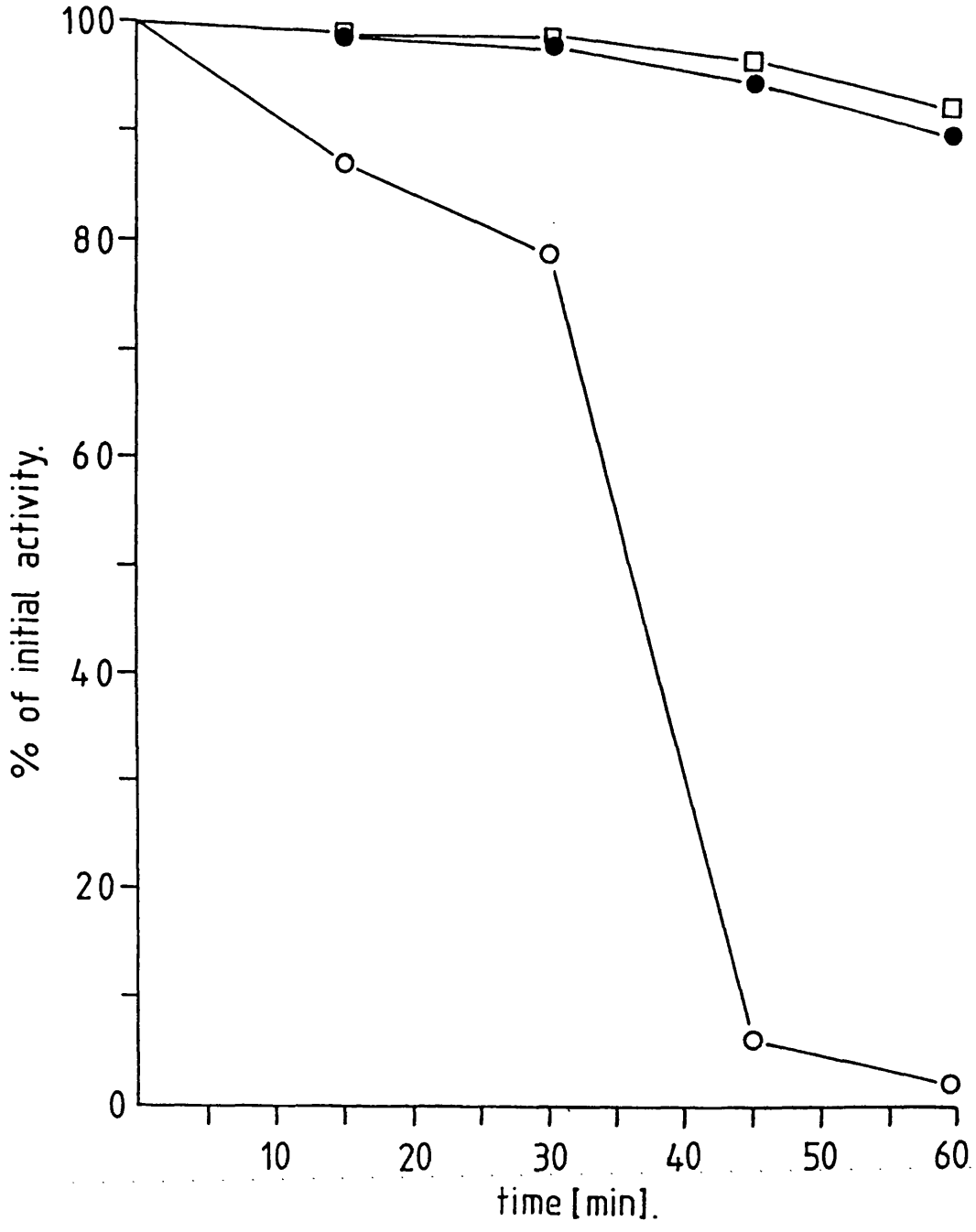


Fig.24 Inhibition of citrate synthase by DTNB.

Inhibition of purified citrate synthase from L. esculentum in the presence and absence of 5mM DTNB. (O——O), citrate synthase from L. esculentum in the presence of 5mM DTNB; (●——●), in the presence of an equivalent volume of buffer; (□—□), pig heart citrate synthase in the presence of 5mM DTNB.

Inhibition of Purified Citrate Synthase

All eukaryotic citrate synthase examined to date are inhibited by adenine nucleotides, normally in the order ATP>ADP>AMP. However, there is doubt concerning the physiological significance of this inhibition and evidence has been presented to show that both the adenine nucleotides and the nicotinamide adenine dinucleotides inhibit non-specifically (Weitzman and Danson, 1976; Weitzman et al., 1978; Weitzman, 1981) probably due to their similarity to the substrate acetyl-CoA (Harford and Weitzman, 1975).

Citrate synthase from L. esculentum was inhibited by ATP and the results are shown in Figs.25 and 26. The inhibition was found to be competitive with respect to acetyl-CoA and non-competitive with respect to oxaloacetate. The enzyme was inhibited to a lesser extent by ADP. No inhibition was detected with NADH between 0.1mM and 1.0mM; however, at a concentration of 10mM, approximately 15% inhibition was detected.

Hirai and Ueno (1977) have reported that citrate synthases from Satsuma mandarin and sweet lime were inhibited by the product of the reaction, citrate. In the present work, citrate, isocitrate and malate were investigated as potential inhibitors of citrate synthase from L. esculentum at concentrations ranging from 50μM to 10mM, but no inhibition was detected.

Malate Dehydrogenase

During the purification of citrate synthase it was noted that two peaks of malate dehydrogenase (MDH) activity were eluted from

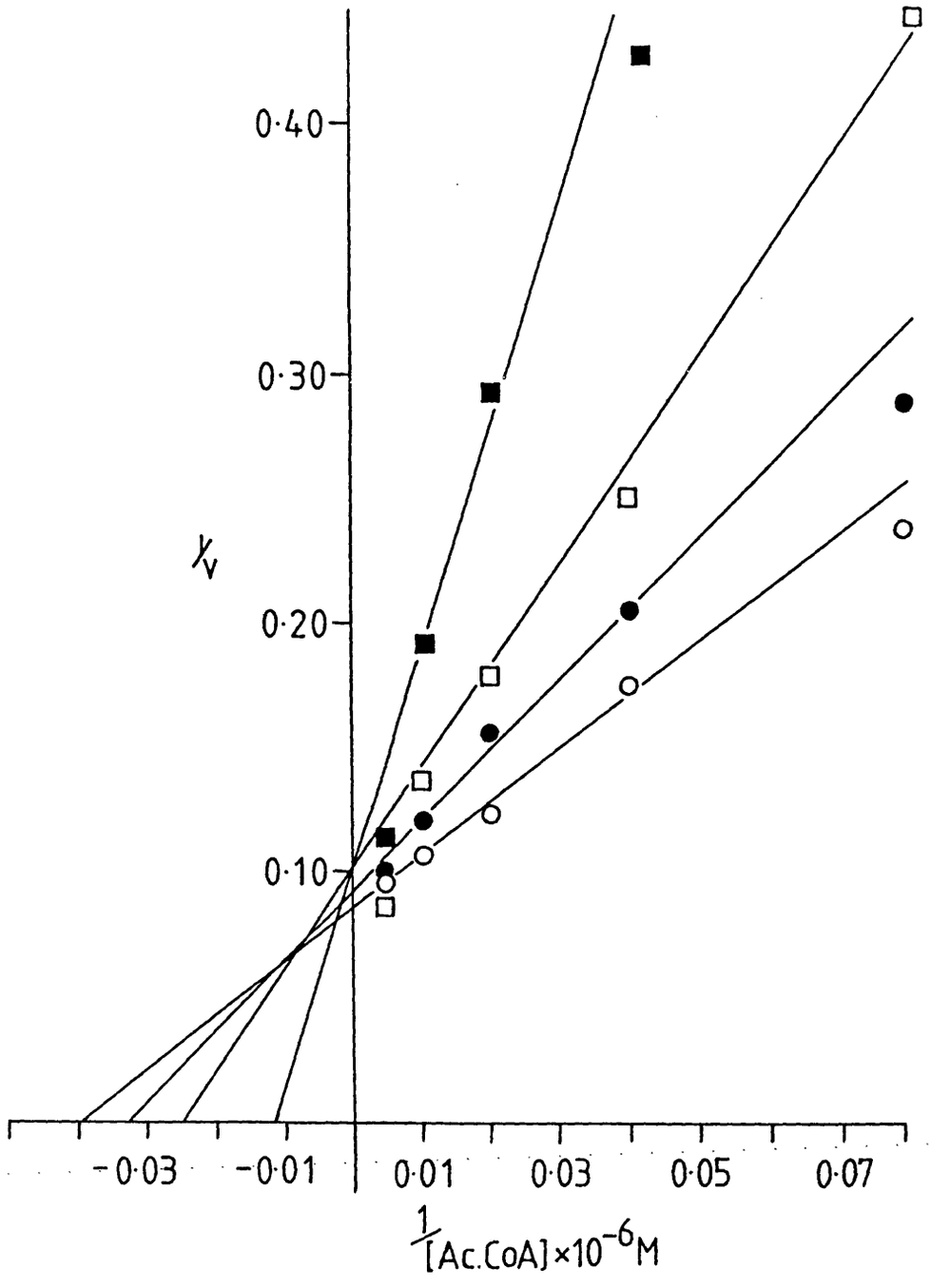


Fig.25 Inhibition of citrate synthase by ATP with respect to acetyl-CoA.

Double reciprocal plot illustrating competitive inhibition of citrate synthase activity by ATP with respect to acetyl-CoA. (O——O), no ATP; (●——●), 50μM ATP; (□—□), 100μM ATP; (■—■), 200μM ATP.

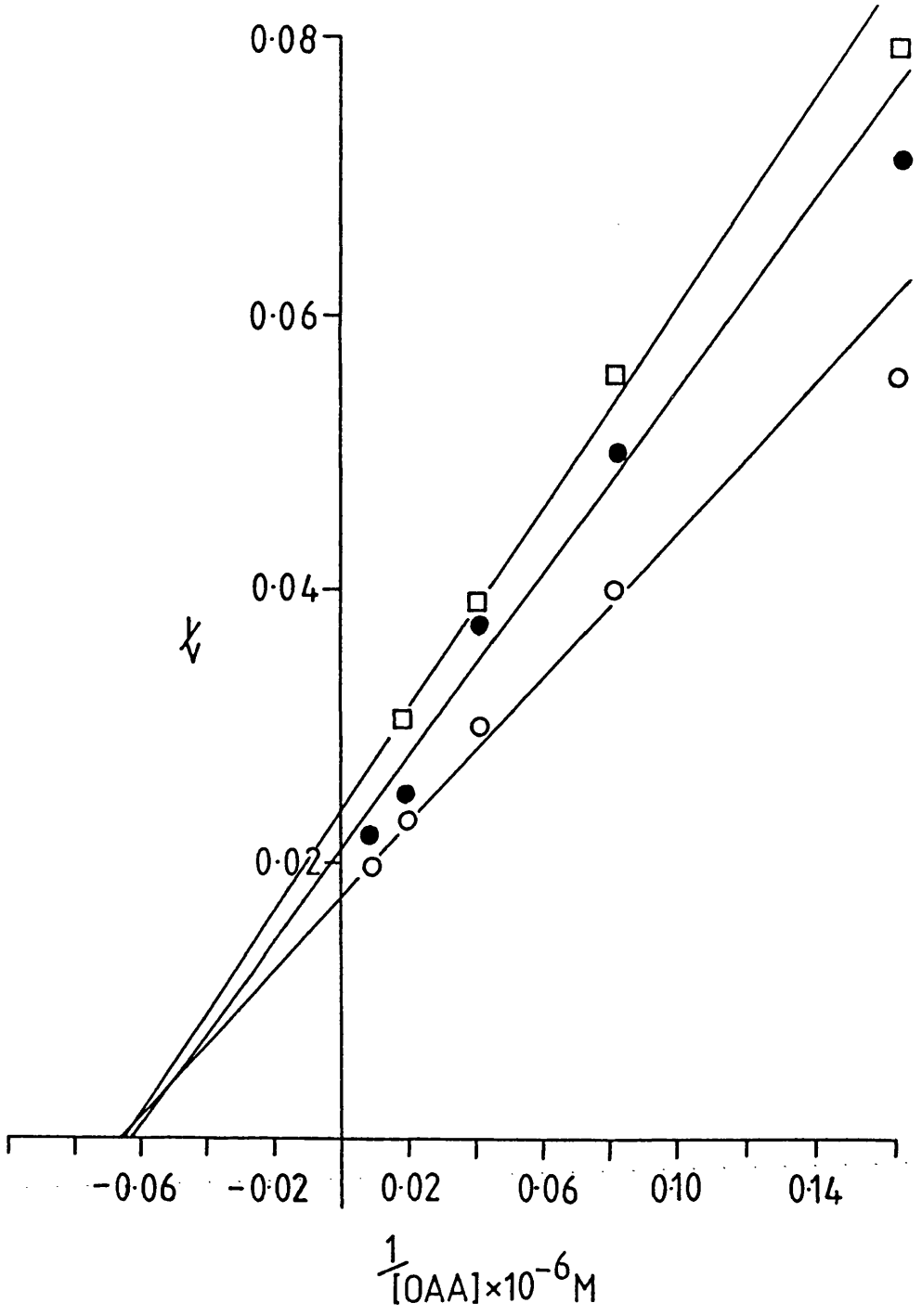


Fig.26 Inhibition of citrate synthase by ATP with respect to oxaloacetate.

Double reciprocal plot illustrating non-competitive inhibition of citrate synthase activity by ATP with respect to oxaloacetate. (O——O), no ATP; (●——●), 100μM ATP; (□—□), 200μM ATP.

Matrex Gel Red A. One peak, which contained 18% of the total activity, co-eluted with citrate synthase at a KCl concentration of 220mM, while the majority of MDH activity eluted at 130mM KCl (Fig.27).

Malate Dehydrogenase that did not co-elute with citrate synthase was further purified by Fast Protein Liquid Chromatography (FPLC) and specific elution from gel Blue A. Malate dehydrogenase (7300 units) was loaded onto a Mono Q FPLC column (equivalent to an anion exchanger) that had previously been equilibrated with 20mM bis, tris propane-HCl, pH 7.4. The column was eluted with a linear sodium chloride gradient from 0-350mM and fractions were collected by peak area. All the activity was recovered in three consecutive fractions. Fractions 17, 18 and 19 contained respectively 1300, 5150 and 850 units. The specific activities of these fractions were:

Fraction 17 1529 units/mg protein

Fraction 18 4682 units/mg protein

Fraction 19 1307 units/mg protein

Five µg of protein from fraction 18 were loaded on a 12.5% SDS polyacrylamide gel and 60µg of protein from the gel Red A peak that included both MDH and citrate synthase were loaded on a separate track. The result for fraction 18 (Fig.28) shows one major band at an approximate M_r of 44,000 and four minor contaminants. The major band from fraction 18 co-migrated with a band on the track containing both MDH and citrate synthase which constituted approximately 10% of the loaded protein.

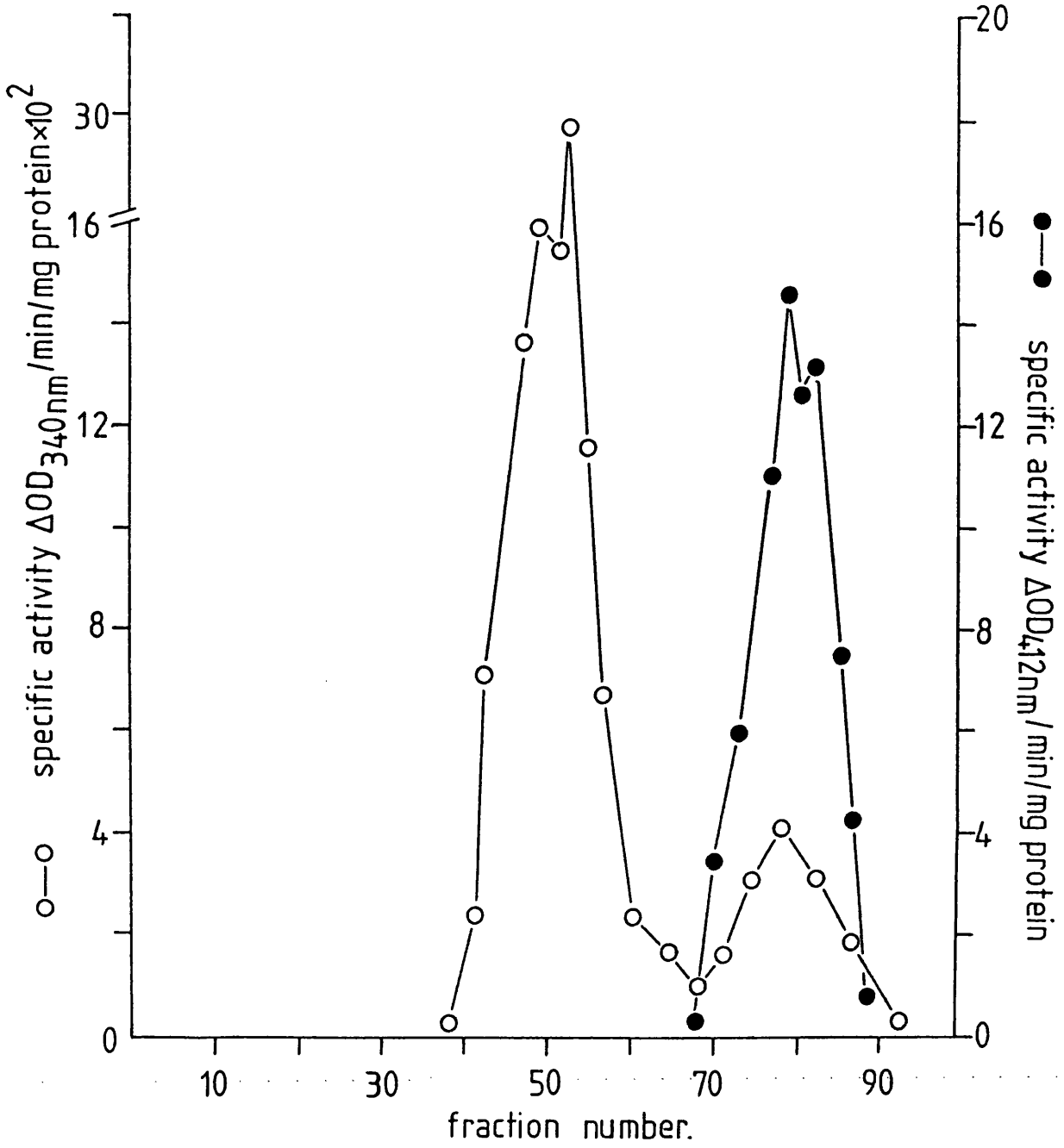


Fig.27 Co-elution of citrate synthase and malate dehydrogenase from Matrex Gel Red A.

Elution profile from Matrex Gel Red A of citrate synthase and malate dehydrogenase from L. esculentum. The salt gradient is not shown, however, citrate synthase (●——●) was eluted at 220mM KCl and malate dehydrogenase (O——O) at 130mM KCl.

Fig.28 Partially purified malate dehydrogenase.

Track 1 illustrates malate dehydrogenase which did not co-elute with citrate synthase from gel Red A and was partially purified by FPLC (fraction 18). Track 2 represents 60µg of protein from the gel Red A peak that included both citrate synthase and malate dehydrogenase activity. Note track 5, which is pepsin from Sigma.

The three FPLC fractions were pooled and dialysed, and 2000 units were loaded on a column of gel Blue A (0.5 x 10cm) which had been equilibrated with 20mM MOPS, pH 8.0. The column was eluted with 1mM malate plus 1mM NADH in the above buffer. MDH activity was found to elute over a long shallow peak of 20ml. However, after concentrating the fractions to 1ml in an Amicon pressure cell, 5µg of protein from this solution were loaded on a 10% SDS polyacrylamide gel. The result (Fig.29) indicates a single band with an estimated M_r of 40,000 (Fig.30). Five µg of this protein were run on a 5% non-denaturing polyacrylamide gel which, unlike the earlier non-denaturing gels, contained a stacking gel. The gel tracks were duplicated and the gel bisected; one half was stained for activity and the other half for protein. The activity stain indicated four bands, one of which was much more intense than the other three. Four protein bands were detected which co-stain with the activity bands (Fig.31).

Malate dehydrogenase (24,000 units) that did not co-elute with citrate synthase from gel Red A were dialysed against distilled water to remove salt and loaded onto a 110ml LKB iso-electric focussing column. After focussing, 1ml fractions were collected and four peaks of activity were detected (Fig.32) with pI values of 4.17, 4,35, 4.50 and 4.70. A similar focussing experiment was conducted with the fraction from gel Red A that contained both MDH and citrate synthase activity. Focussing revealed one peak of activity with a pI of 4.17. During the iso-electric focussing, 95% of the citrate synthase activity was lost, while on both occasions more than 90% of the MDH activity was recovered. The M_r of MDH was estimated to be

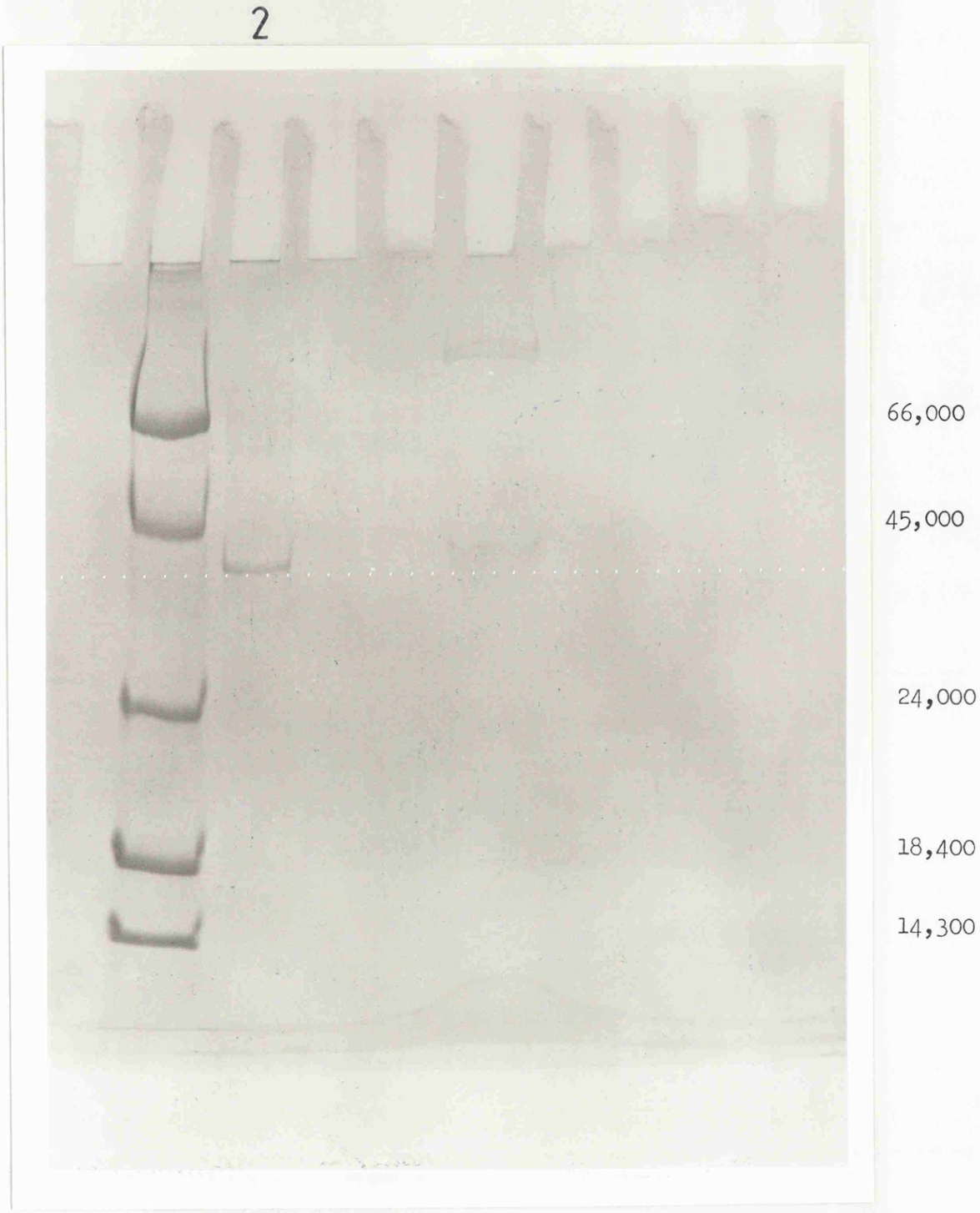


Fig.29 Purified malate dehydrogenase.

A 10% SDS slab gel illustrating pure malate dehydrogenase from L. esculentum in track 2. 5µg of malate dehydrogenase were loaded and 10µg of each marker.

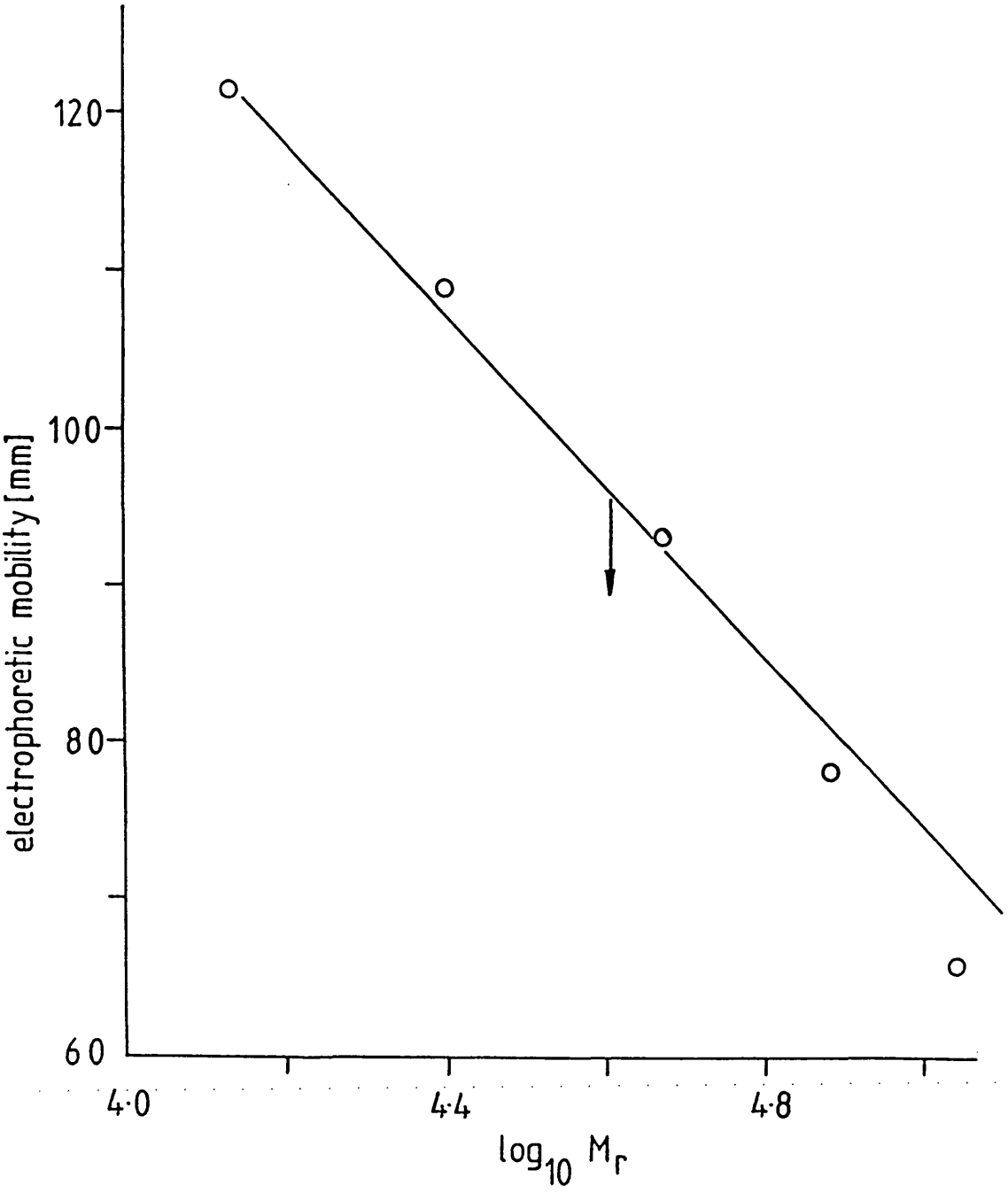


Fig.30 Estimation of malate dehydrogenase sub-unit M_r.

Plot of electrophoretic mobility against $\log_{10}M_r$ of marker proteins used in the determination of the sub-unit size of malate dehydrogenase from L. esculentum. From left to right marker proteins are; lysozyme (14,300), β-lactoglobulin (18,400), trypsinogen (24,000), ovalbumin (45,000) and BSA (66,000). The arrow indicates the approximate $\log_{10}M_r$ of the malate dehydrogenase sub-unit.

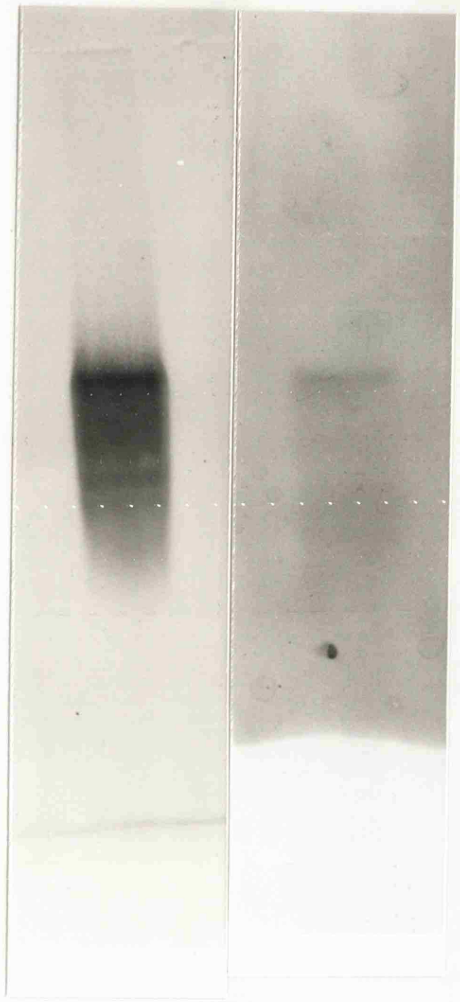


Fig.31 Resolution of malate dehydrogenase isoenzymes on non-denaturing polyacrylamide gel.

Native polyacrylamide slab gel illustrating the four isoenzymes of malate dehydrogenase. The activity stain is on the left and the protein stain on the right. Five µg of protein were loaded on each track.

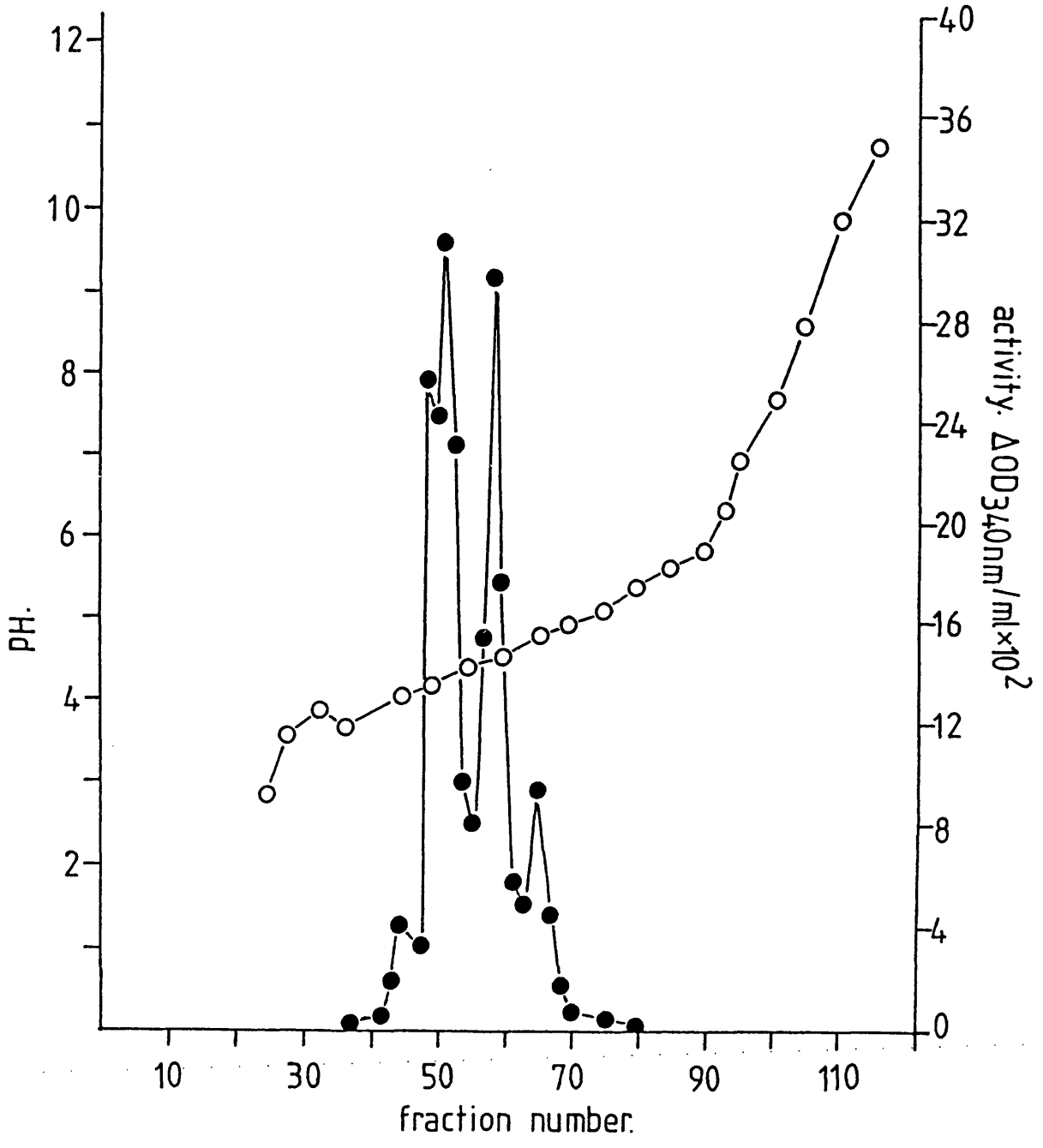


Fig.32 Resolution of malate dehydrogenase isoenzymes by isoelectric focussing.

Elution profile of malate dehydrogenase isoenzymes from an LKB isoelectric focussing column (110ml). The pH gradient is shown by the open circles and malate dehydrogenase activity by the closed circles. 24,000 units were added in a total of 2ml to the light solution and 22,780 recovered. Initially, 500V were applied to the column; this was gradually increased to 2,000V. Focussing was completed when the current stabilised at its lowest level.

76,000 by gel permeation chromatography using Sephadex G-100 in 50mM MOPS, pH 8.0 (Fig.33). Marker proteins used were: aldolase (158,000), bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen (25,000) and ribonuclease A (13,600). Proteins were detected with a 280nm light source which was connected to an LKB chopper bar recorder.

Detection of NAD-Isocitrate Dehydrogenase, NAD-Malic Enzyme and Pyruvate and α-Oxoglutarate Dehydrogenase in L. esculentum

One of the main problems encountered in assaying NAD-dependent isocitrate dehydrogenase, NAD-dependent malic enzyme and pyruvate and α-oxoglutarate dehydrogenases, is that they are all large oligomeric regulatory proteins and are therefore susceptible to damage during extraction. The oxoacid dehydrogenase complexes are especially prone to damage, although they normally retain their dihydrolipoamide dehydrogenase (E3) activity. Additionally, tomato fruit contain relatively small amounts of protein (supernatants contain approx. 1mg/ml) which means that there is not much activity to start with. Tomato supernatant also contain an active NADH oxidase which interferes with NAD-linked enzyme assays.

The first method attempted was to inactivate the NADH oxidase with 30mM cyanide. However, the cyanide must be removed as it would interfere with the pyruvate dehydrogenase assay by preventing the reduction of NAD^+. Mature green Sonatine (80g) were extracted as described and potassium cyanide was added to 3ml of the resulting supernatant to give a final concentration of 30mM. The solution was loaded onto a column of Sephadex G-25 (1 x 15cm)

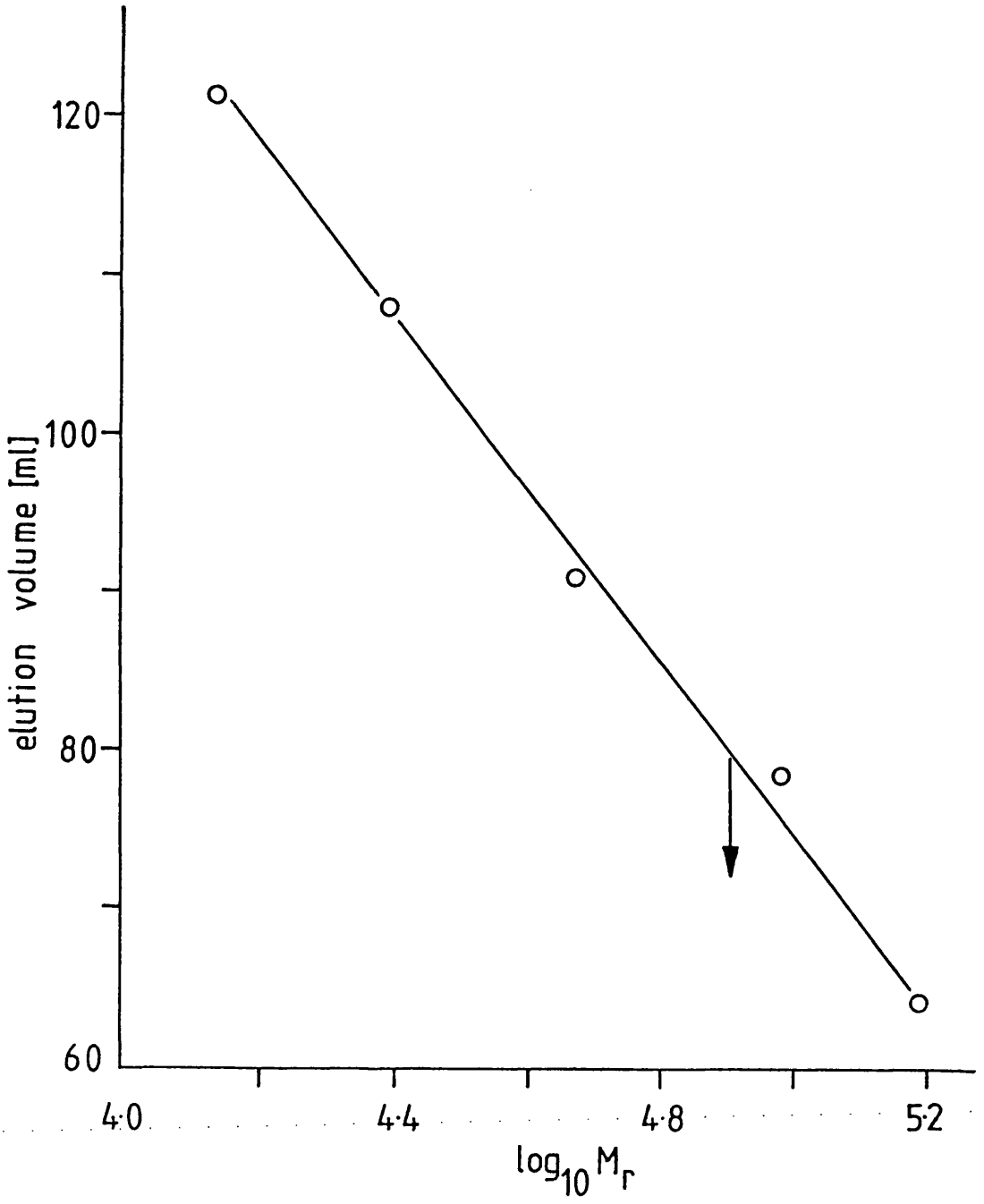


Fig.33 Estimation of malate dehydrogenase M_r.

Plot of elution volume against $\log_{10}M_r$ for Sephadex G-100 marker proteins. Results are the mean of three determinations in which the elution volumes for each protein varied less than 0.5ml. From left to right the marker proteins are: ribonuclease A (13,600), chymotrypsinogen (25,000), ovalbumin (45,000), BSA (68,000) and aldolase (158,000). The arrow indicates the approximate $\log_{10}M_r$ for malate dehydrogenase from L. esculentum.

equilibrated in 50mM phosphate buffer, pH 8.0. The solution contained a few micrograms of Dextran Blue and riboflavin which acted as visual markers, thus allowing rapid detection of proteins. MDH was used to detect activity, which was eluted in the first 3ml after the Dextran Blue. No NADH oxidase activity was detected in these fractions but neither was any pyruvate or α-oxoglutarate dehydrogenase. The experiment was repeated after concentrating the supernatant by one of two methods:

1. By fractionating the supernatant with 80% ammonium sulphate and resuspending the resulting pellet in a minimum volume of buffer.
2. By enclosing the supernatant in dialysis tubing and removing water by evaporation at 4°C in a forced air stream.

In neither case was any activity detected. It was decided to assay for dihydrolipoamide dehydrogenase (E3) activity. However, the substrate dihydrolipoate requires alcohol for solubility and the supernatant from tomato extract contains an active alcohol dehydrogenase which again interfered with the assay. In fact pyruvate dehydrogenase activity was not detected until mitochondria were purified and concentrated by sub-cellular fractionation later.

NAD-Isocitrate Dehydrogenase and NAD-Malic Enzyme

Cox and Davies (1967) showed that NAD-linked isocitrate dehydrogenase extracted from pea mitochondria was inhibited by chloride ions. They demonstrated that, at 75% saturating substrate concentration, a buffer containing 50mM chloride ions would cause 60% inhibition of enzyme activity. This was another reason for

changing from tris-HCl to MOPS, although, at the time, it made little difference to the detection of NAD-linked isocitrate dehydrogenase. Hitherto, the existence of NAD-linked isocitrate dehydrogenase in L. esculentum has not been demonstrated. Although Hobson (1974) detected isocitrate dehydrogenase by staining for activity on polyacrylamide gels, he did not state whether the activity was NAD- or NADP-dependent.

The methods used in attempting to detect pyruvate and α-oxoglutarate activity were employed for NAD-linked isocitrate dehydrogenase and NAD-linked malic enzyme, again without success. It was therefore decided to attempt to detect activity by staining, using the method of Harris and Hopkinson (1976). Two ml of supernatant extracted from mature green Sonatine were passed through a small column of Sephadex G-25 to remove any salts that might interfere with electrophoresis; 100µl of the supernatant were mixed with the same volume of dissolving buffer and loaded onto 5% non-denaturing polyacrylamide gels. The gels were run in the cold room at 3mA per gel and, after electrophoresis, were stained for malate dehydrogenase, NAD-dependent isocitrate dehydrogenase and NADP-dependent isocitrate dehydrogenase activities. The usual precautions were taken to keep solutions of MTT and PMS away from the light. Both MDH and NADP-dependent isocitrate dehydrogenase stained for activity, the former within 5min of staining and the latter within 30min. There were 4 distinct bands of MDH activity and 2 of NADP-dependent isocitrate dehydrogenase. No stain appeared for NAD-dependent isocitrate dehydrogenase activity.

It was decided at this point to assay for NAD-dependent isocitrate dehydrogenase in a plant tissue in which it had already been detected. The tissue chosen was swede, from which the enzyme had been successfully extracted (Dennis and Coultate, 1967). The published method was followed, except that 50mM MOPS was substituted for 50mM tris and the sucrose content of the buffer was reduced from 700mM to 300mM. NAD-dependent isocitrate dehydrogenase activity was detected in the resuspended acetone powder of the crude mitochondrial pellet and the activity was linear with respect to enzyme concentration between 17.5 - 70µl of extract. No activity was found in the supernatant; however, NADP-dependent isocitrate dehydrogenase activity was detected in the supernatant and, in terms of rate, was an order of magnitude greater than in L. esculentum.

Having extracted the enzyme from swede, the same procedure was applied to tomato fruit. It was thought advantageous, however, to sonicate the crude mitochondrial pellet rather than make an acetone powder. The pellet was therefore divided into two; one half was sonicated for three 15sec bursts at 15 watts output and the other half converted into an acetone powder. No NAD-dependent isocitrate dehydrogenase activity was detected in the sonicated portion, whereas activity was detected in the resuspended acetone powder. The enzyme was found to be extremely labile, losing 50% activity in 30min; the pH optimum was 7.6. NAD-dependent malic enzyme activity was also detected in the same acetone powder extract. However, when aliquots from the crude mitochondrial pellet were incubated in the presence of 0.03% Triton X-100, greatly increased rates for both enzymes were demonstrated. Both enzymes had an

absolute requirement for a divalent cation and Mn^{2+} was used in all assays. The pH optimum for NAD-dependent malic enzyme was found to be pH 8.0. This differs markedly from the pH values for NAD-dependent malic enzyme from cauliflower of 6.9 (Macrae, 1971) and 6.4 (Davies and Patil, 1975). No rate was detected for NAD-dependent malic enzyme from L. esculentum when either Mn^{2+} or malate were absent from the assay. However, in an attempt to determine that NAD-dependent malic enzyme was being measured and not MDH, it was decided to determine the product of the reaction - pyruvate. The malic enzyme reaction was allowed to go to completion at pH 8.0 and 5 units of lactate dehydrogenase were added to each of the control and reaction cuvettes. NADH oxidation occurred that was stoichiometric with the amount of NAD^+ reduced in the initial reaction. However, when 5 units of the same lactate dehydrogenase were added to a cuvette containing buffer, 0.2mM oxaloacetate and 0.4mM NADH, the oxidation of NADH occurred, albeit at a slower rate than that observed in the initial assay. This indicated that the lactate dehydrogenase was contaminated with MDH activity. The contaminating MDH activity was removed by FPLC and the initial assay repeated, with the same result; this confirmed that the product was indeed pyruvate.

Since it was now possible to assay the majority of enzymes that were directly involved in citric and malic acid metabolism, it was decided to investigate their exact cellular location. This would serve several purposes:

1. It would provide concrete evidence for the site of organic acid synthesis.

2. Definitive evidence could be obtained regarding the existence of the glyoxylate cycle within ripening tomato fruit (Doyle and Wang, 1960).
3. It would provide further data on the existence of isoenzymes of citrate synthase or any of the other enzymes under study.

Sub-Cellular Fractionation of Fruit from L. esculentum using Percoll Gradients

Percoll is a heterogeneous silica sol; the particles range from 13-30nm and are coated with PVP. Percoll has several advantages over sucrose as a gradient medium: (a) it is iso-osmotic throughout the gradient; (b) because of its low viscosity, low spin speeds and low centrifugal forces can be employed; (c) it can form self-generated gradients within the tube; (d) it is unable to penetrate biological membranes. These, plus the fact that pre-formed gradients can be stored almost indefinitely, make Percoll an attractive gradient medium.

Gradient Standardisation

Percoll was made iso-osmotic with 2.5M sucrose and then diluted with Percoll dilution buffer in the following ratios. All figures are in ml.

Percoll	35	31.5	28	24.5	21	17.5	14	10.5	7	3.5
Dilution Buffer	0	3.5	7	10.5	14	17.5	21	24.5	28	31.5

Ten µl of each density bead were loaded onto each gradient and the gradients spun at 40,000g_{max} for 25min. A plot of bead density

against bead R_f values is shown in Fig.34 and a pictorial display is illustrated in Fig.35.

Preparation of Gradient Samples

Several methods were employed to break open the cells and extract intact organelles. The initial methods all used some form of mechanical shearing device to homogenise the fruit and break open the cells. However, although the results improved with practice, the best method never achieved respiratory control ratios better than two. Eventually, a modification of the method of Hobson (1969) was used. Although extremely tedious, especially when using small fruit, the method gave excellent results with tightly coupled mitochondria (Fig.36).

Three ml of the resuspended crude mitochondrial pellet from mature green Sonatine, containing approximately 18mg of protein, were loaded onto a 60% Percoll gradient; the tube was inverted several times and then spun at 40,000g_{max} for 30min. The result showed no separation of mitochondria or chloroplasts within the gradient. Several gradients were spun for longer periods, or at faster speeds, but all with the same results. Eventually, it was decided to fractionate the mitochondria and chloroplasts on separate gradients.

Mitochondrial Fractionation

A differential centrifugation designed to separate the majority of the chloroplasts from mitochondria was devised (see Methods p.35, Fig.37). Crude mitochondrial protein (18 - 20mg) was loaded onto a

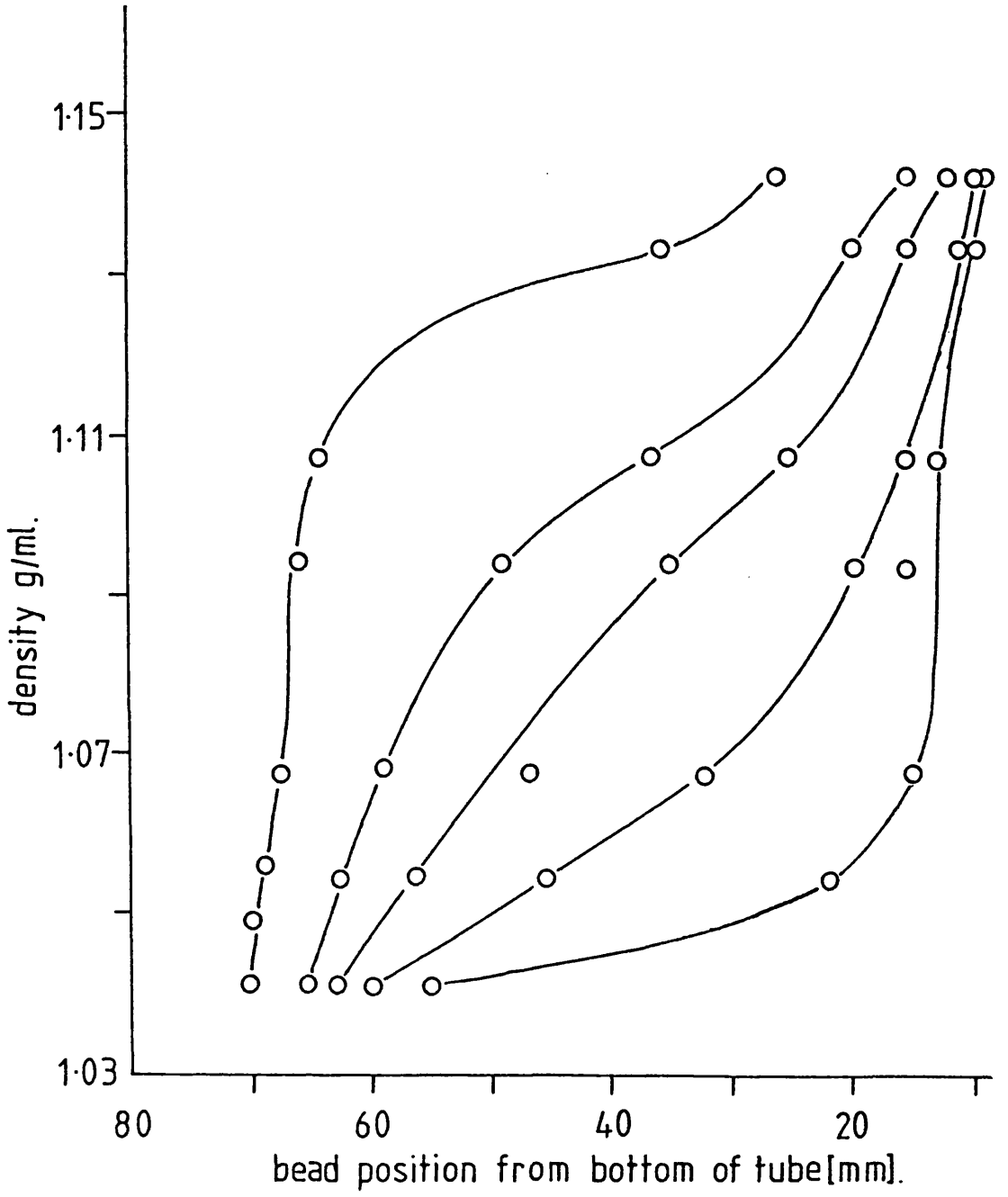


Fig.34 Diagrammatic representation of Percoll gradient standardisation.

Plot of bead migration distance against bead density of Percoll internal gradient marker beads for a series of different gradients. From left to right the amount of iso-osmotic Percoll to dilution buffer is as follows: 90%, 70%, 60%. 50% and 30%. For details see Methods, p.36.

Fig.35 Pictorial display of Percoll gradient standardisation.

Pictorial display of Percoll gradient standardisation. 10µl of suspension containing beads of different density were loaded onto each gradient. From left to right: 20% - 90% Percoll in 10% steps. Bead density varied from 1.019 to 1.139g/ml. Gradients were spun at 40,000g for 30min.

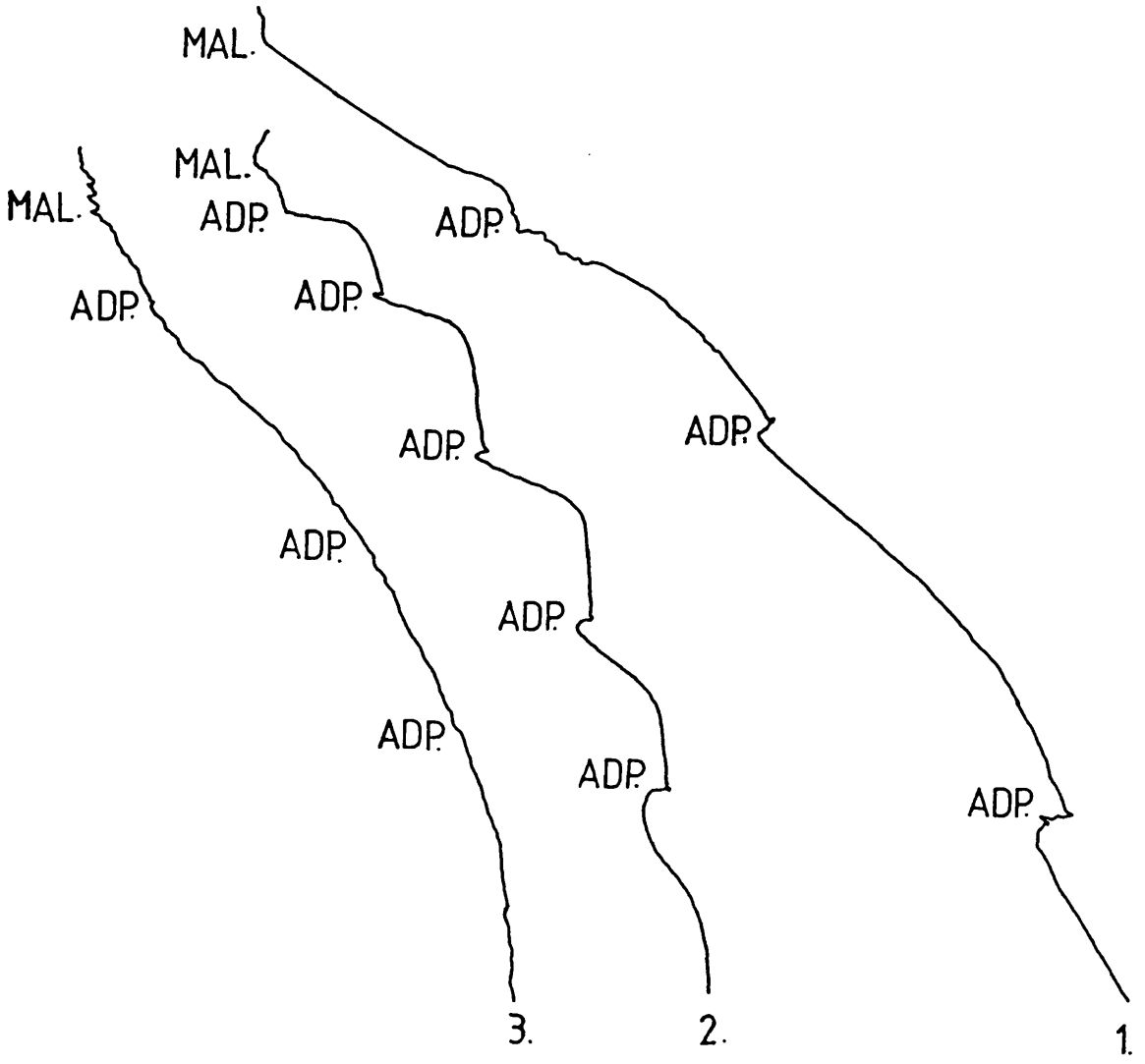


Fig.36 Mitochondrial oxygen uptake.

Traces of polarographic recordings from a Clark type oxygen electrode of oxygen uptake by mitochondria, extracted from mature green Sonatine. Trace 1, mitochondria extracted using a Braun or Waring blender. Trace 2, mitochondria extracted using a cheese grater. Trace 3, mitochondria extracted using a cheese grater but without BSA in the extraction buffer. Final concentrations of malate and ADP were 17mM and 0.5mM respectively. Oxygen content of the medium was 240μmoles/l and the final volume of the reaction mixture was 2.3ml.

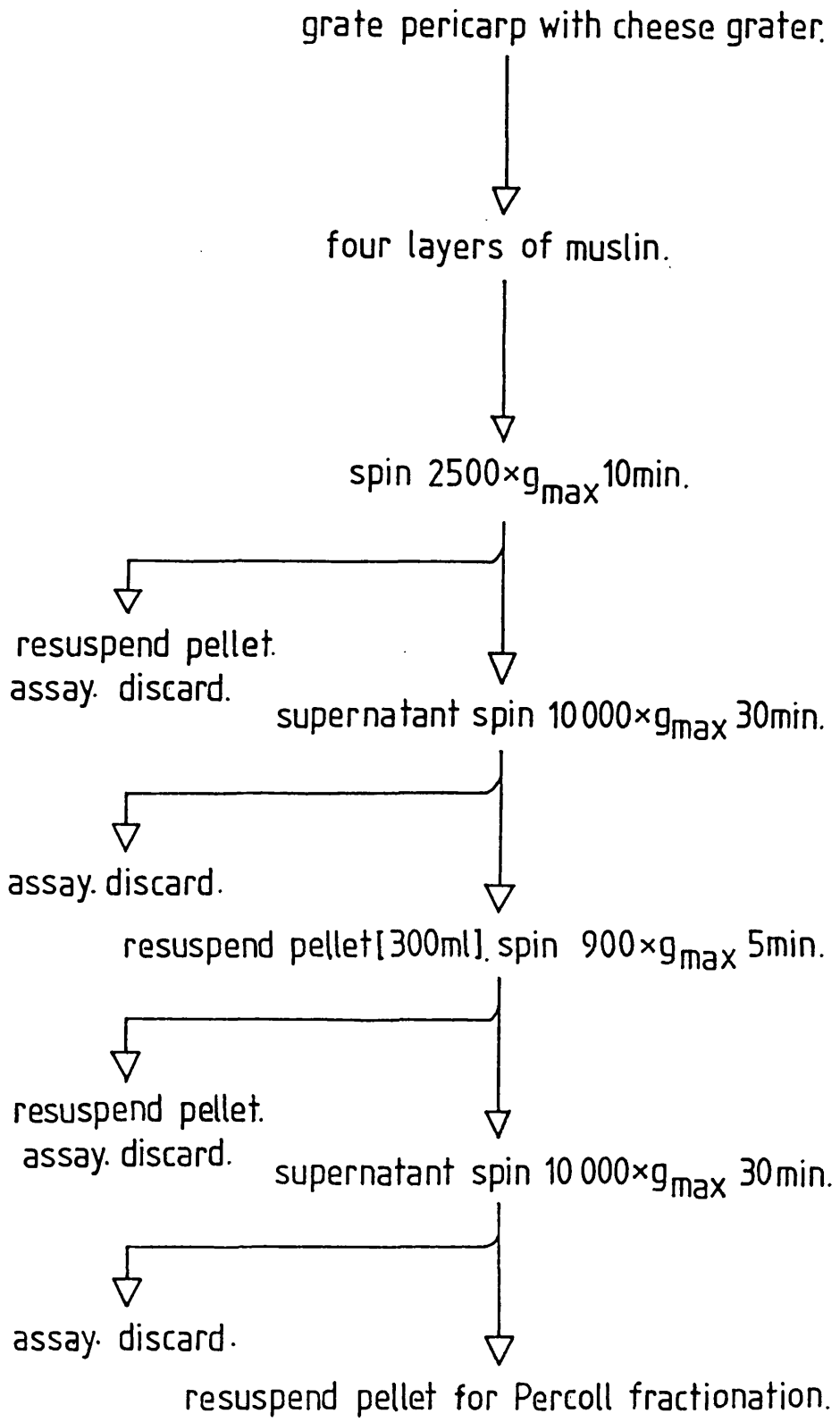


Fig.37 Protocol for preparation of pericarp tissue prior to sub-cellular fractionation.

stepped Percoll gradient and centrifuged at 12,000 x g_{max} for 30min. The gradient was originally composed of 13.5/21/45% discontinuities. This gave a starting point from which to devise better gradients and spin times since it was possible to see the buff-coloured mitochondrial band at the interface between the 21/45% discontinuities. However, on fractionating the gradient, it was obvious that the mitochondria had not all reached the interface since succinate dehydrogenase activity was distributed throughout the gradient. The discontinuities were eventually increased to 13.5/28/55% Percoll and the spin time increased to 60min. These alterations yielded gradients that gave reproducible results (Fig.38). Fractionation of these gradients showed that the microbody fraction had penetrated and banded in the 55% discontinuity, while the mitochondria still banded at the interface between the 28/55% steps. There was a clear gap before the lipid and chlorophyll/broken chloroplast fraction could be seen. Spectrophotometric analysis of the fractions (Figs.39 and 40) showed the clear separation of glycollate oxidase from succinate dehydrogenase, and revealed the mitochondrial localisation of NAD-linked isocitrate dehydrogenase, NAD-linked malic enzyme and citrate synthase.

Chloroplast Fractionation

The protocol for the differential centrifugation was altered slightly for chloroplast fractionation. After grating, the homogenate was spun rapidly up to 8,000 x g_{max}, then brought to rest as quickly as possible. The chloroplasts were gently removed from the top of the pellet using a camel hair brush and loaded onto a continuous

Fig.38 Mitochondrial purification on a Percoll gradient.

Fractionation of a crude enriched mitochondrial/microbody fraction from mature green Sonatine on a discontinuous Percoll gradient of 13.5/28/55% Percoll. 35mg of protein were loaded in 3ml of extract.

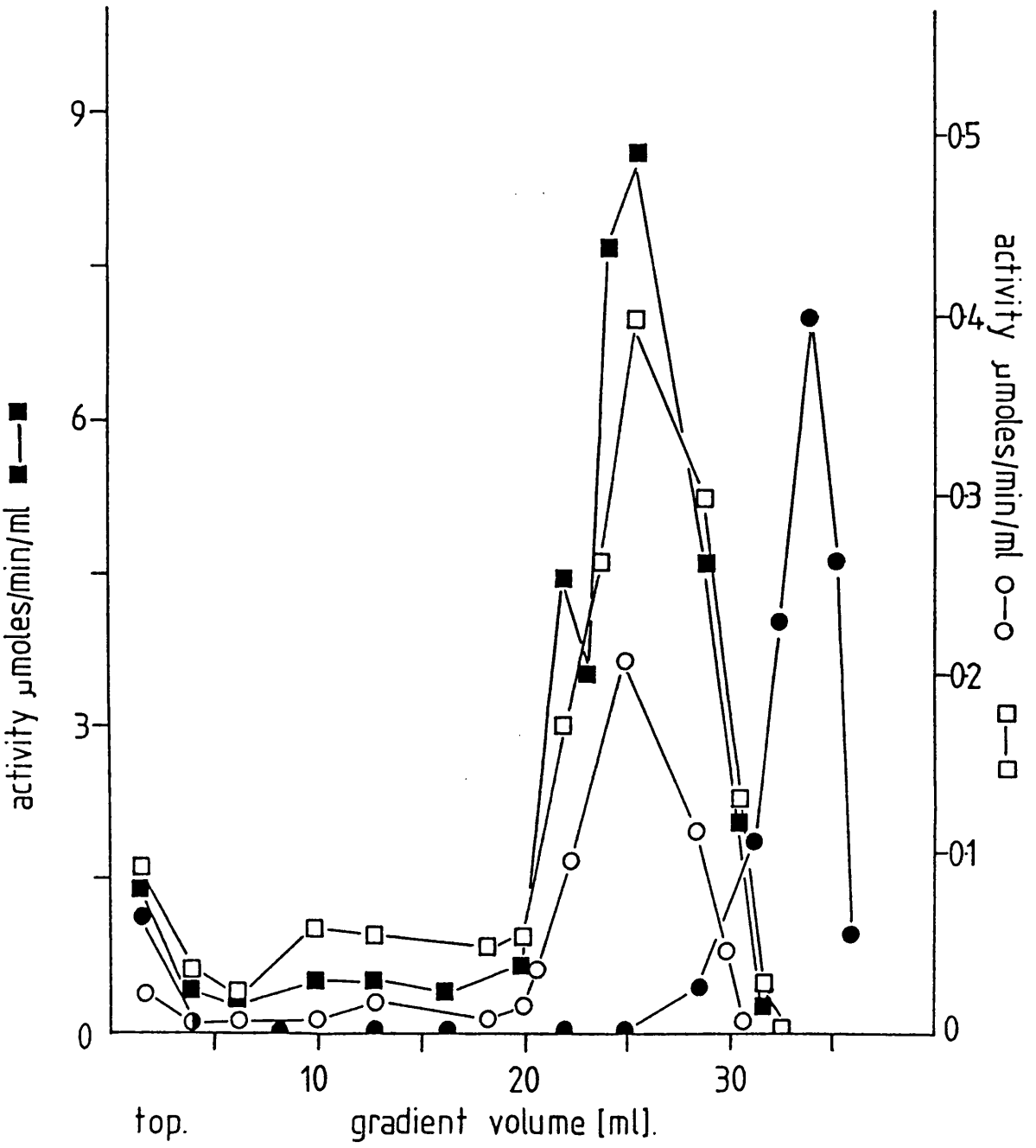


Fig.39 Localisation of enzyme activity (succinate dehydrogenase, glycollate oxidase, citrate synthase and malate dehydrogenase) using a discontinuous Percoll gradient.

Fractionation on a discontinuous gradient (13.5/28/55% Percoll) of an enriched mitochondrial/microbody pellet, extracted from mature green Sonatine. Enzymes are: (○——○), succinate dehydrogenase; (●——●), glycollate oxidase; (□—□), citrate synthase and (■—■), malate dehydrogenase. Recoveries were: MDH 84%, citrate synthase 110%, glycollate oxidase 75% and succinate dehydrogenase 109%. Scale for glycollate oxidase is not shown but ranged from 0.05 - 0.25µmoles/min/ml.

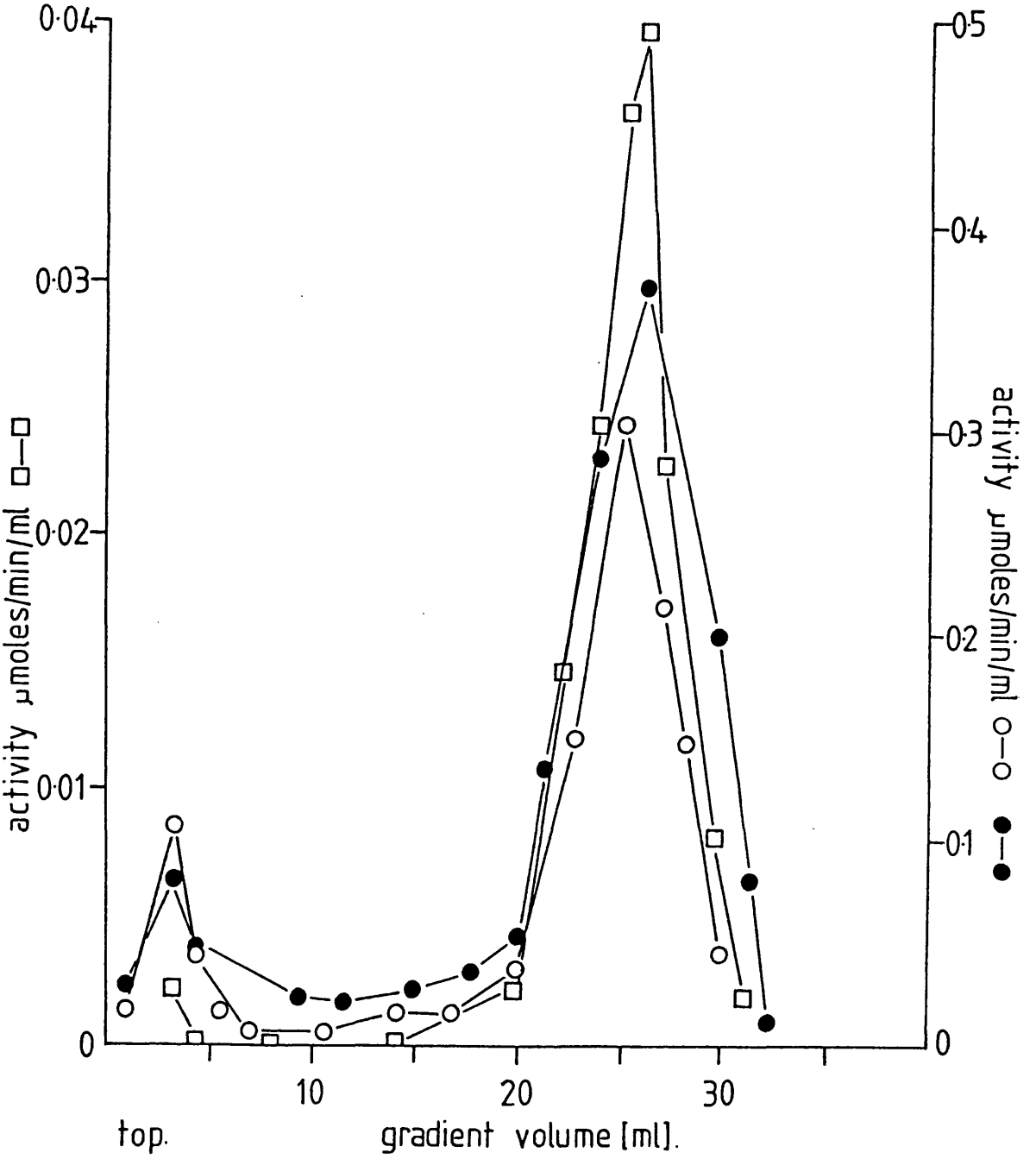


Fig.40 Localisation of enzyme activity (NAD-linked malic enzyme, citrate synthase and NAD-linked isocitrate dehydrogenase) using a discontinuous Percoll gradient.

Fractionation on a discontinuous (13.5/28/55% Percoll) gradient of an enriched mitochondrial pellet from mature green Sonatine. The marker enzymes are: (o——o), NAD-linked malic enzyme; (●—— ●), citrate synthase and (□—□), NAD-linked isocitrate dehydrogenase. Recoveries were: citrate synthase 86%, NAD-linked malic enzyme 92% and NAD-linked isocitrate dehydrogenase 94%.

15 - 90% Percoll gradient. A control gradient containing the internal density marker beads was run to determine the buoyant density of intact chloroplasts. The gradient was spun at 12,000 x g_{max} for 40min. The result (Fig.41) shows 2 distinct bands at buoyant densities of 1.040g/ml and 1.095g/ml. After fractionation, the chloroplast marker enzyme NADP-linked glyceraldehyde 3-phosphate dehydrogenase was found only in the band with the higher buoyant density. As shown in Fig.42, citrate synthase activity was absent from the band containing intact chloroplasts, as was NAD-linked isocitrate dehydrogenase. Although not shown, no activity was detected for NAD- or NADP-linked malic enzyme. In addition, no activity for any of the enzymes under study could be detected in the absence of 0.03% triton in the assay mixture. This was true for all but the top 4 - 5ml of the gradient, since these fractions contained enzymes from organelles that had burst open.

Examination of Isolated Mitochondria

Mitochondria isolated in Percoll gradients from the remainder of the cellular constituents retained their integrity as judged from latency experiments for periods exceeding one week, and the activity of citrate synthase within the mitochondria fell by less than 3% during the same period (mitochondria from mature green fruit). The intactness of mitochondria isolated from Percoll gradients is illustrated in Fig.43. Their purity can be judged from the results of a continuous scan between 704 - 308nm of an acetone extract from purified mitochondria in comparison with a similar extract from the

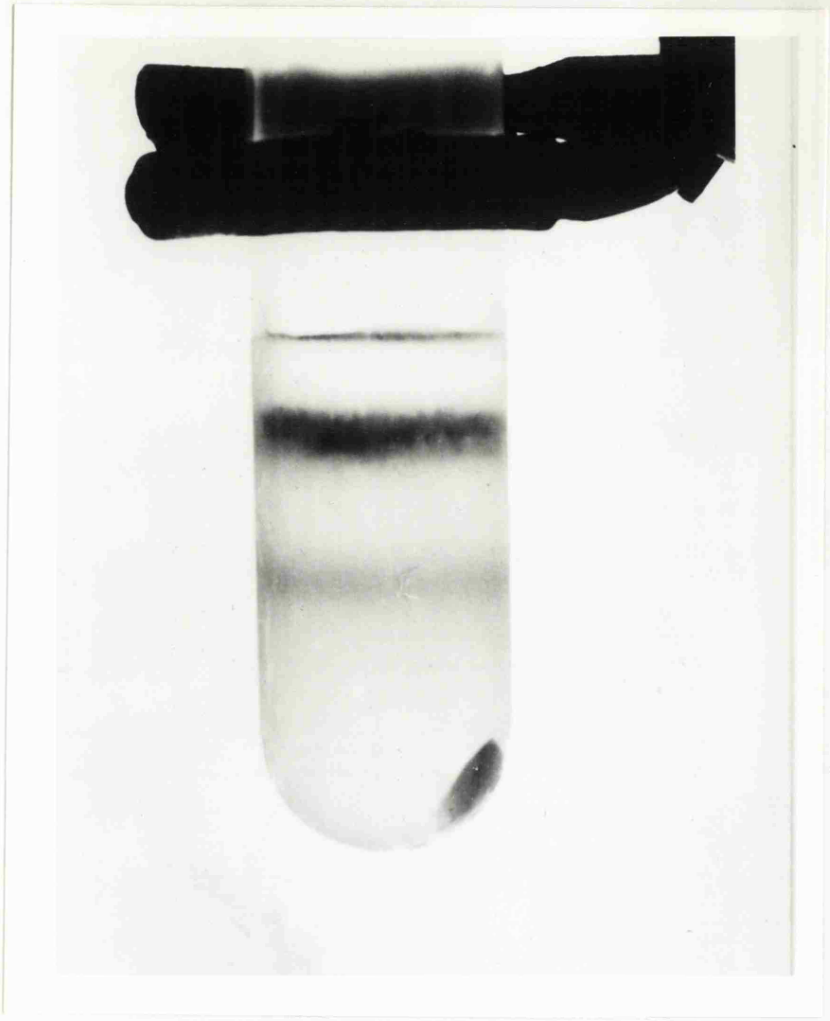


Fig.41 Chloroplast purification on a Percoll gradient.

Fractionation of a crude enriched chloroplast pellet from mature green Sonatine on a 15 - 90% continuous Percoll gradient. The gradient was spun at 12,000 x g_{max} for 40min. 20mg of protein were loaded in 1.8ml of extract.

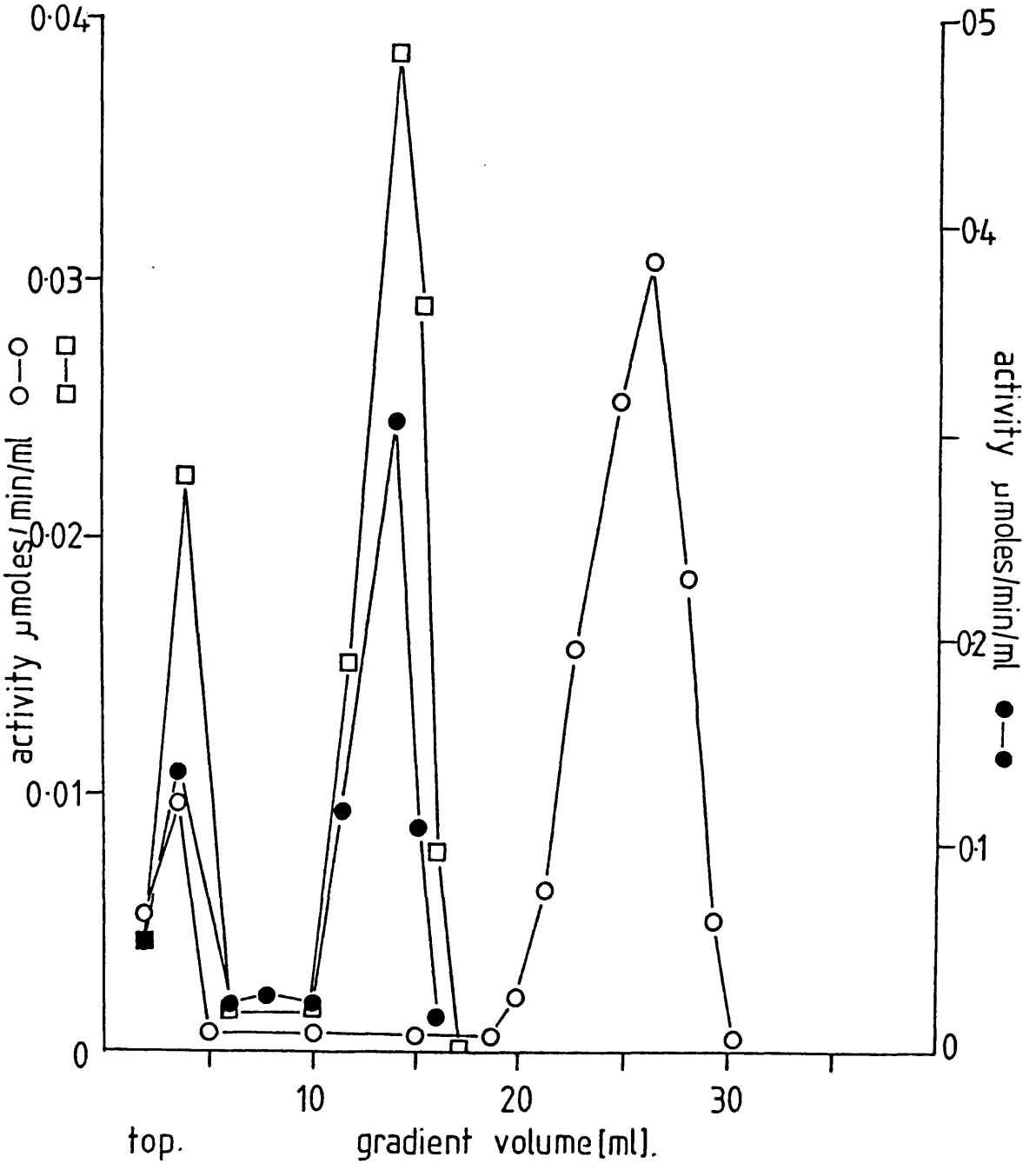


Fig.42 Localisation of enzyme activity (NADP-linked glyceraldehyde 3-phosphate dehydrogenase, citrate synthase and NAD-linked isocitrate dehydrogenase).

Fractionation on a continuous 15 - 90% Percoll gradient of an enriched chloroplast pellet extracted from mature green Sonatine. The marker enzymes are: (O——O), NADP-linked GAPDH; (●——●), citrate synthase and (□—□), NAD-linked isocitrate dehydrogenase. Recoveries were: citrate synthase 88%. NAD-linked isocitrate dehydrogenase 92% and NADP-linked GAPDH 102%.

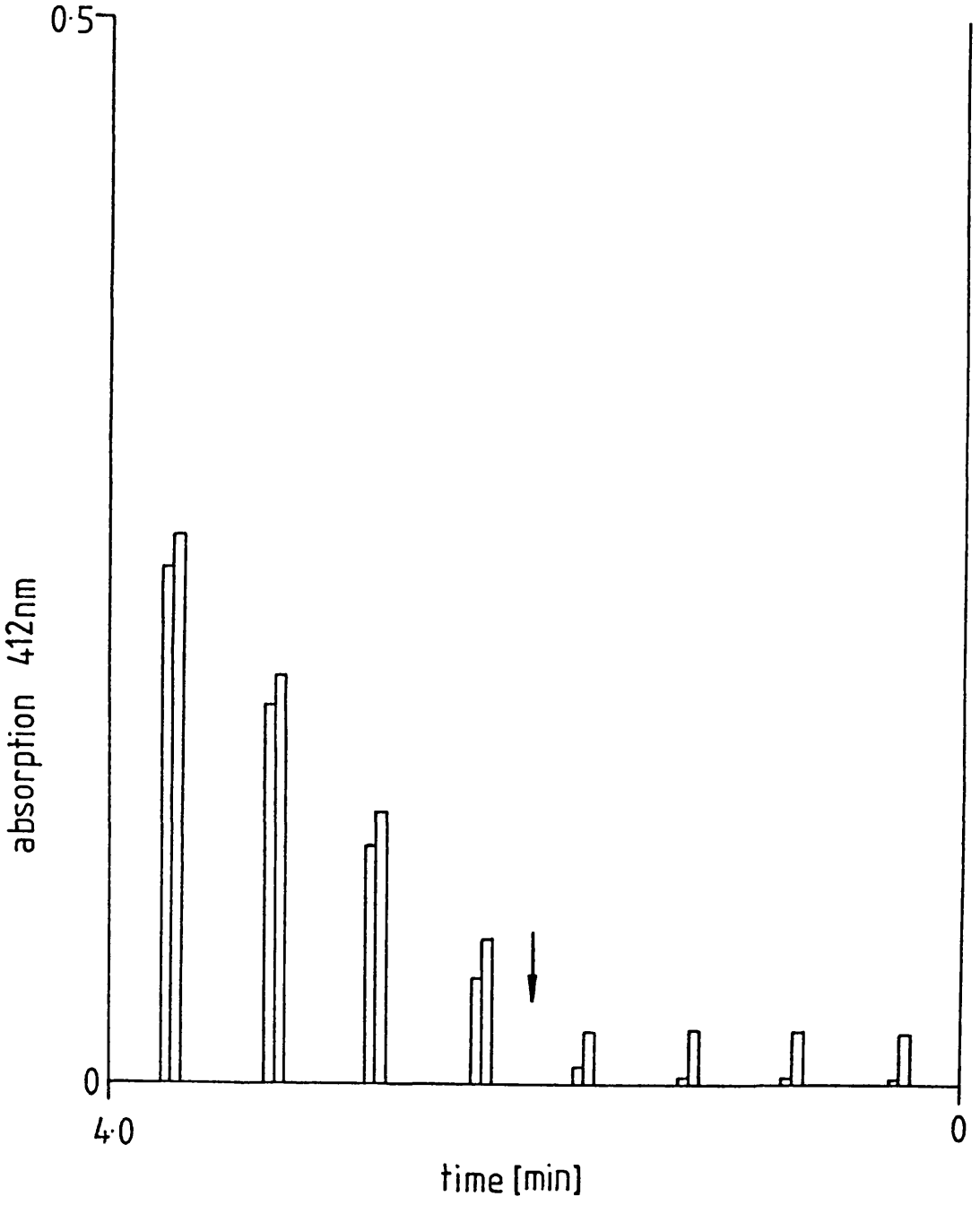


Fig.43 Latency of enzyme activity in Percoll purified mitochondria.

Spectrophotometric trace illustrating the intactness of Percoll purified mitochondria by enzyme latency. The enzyme assayed was citrate synthase and the time interval between each duplicate assay was 0.5min. The arrow indicates the time at which Triton X-100 was added to the assay at a final concentration of 0.03% ($^{v}/_{v}$).

crude mitochondrial pellet (Fig.44). Percoll-purified mitochondria revealed respiratory control and ADP/O ratios comparable with those found in the crude mitochondrial pellet.

The importance of isolating mitochondria is illustrated by the spurious activities of NADP-dependent isocitrate dehydrogenase and NADP-dependent malic enzyme found in the crude mitochondrial pellet. Approximately 5% of the total activity of both enzymes was recovered in the crude mitochondrial pellet. However, when mitochondria were purified on Percoll gradients, no activity could be found.

Malate dehydrogenase was the only enzyme under study that was not exclusively mitochondrial, but was distributed between the cytosol and the mitochondria. In mature green fruit, the ratio of mitochondrial to cytosolic MDH was normally 14/75, expressed as percentages of the total activity in the homogenate, the remaining 11% was associated with the first pellet. Isocitrate lyase activity was not detected in any fraction, including the microbody fraction.

Changes in Specific Activity of Citric Acid Cycle Enzymes from Percoll-Purified Mitochondria at Different Stages of Ripening

Two hundred grams of mature green Sonatine were extracted in 600ml of mitochondrial extraction buffer as described. Each fraction of the differential centrifugation was assayed for citrate synthase, MDH, NAD-dependent isocitrate dehydrogenase and NAD-dependent malic enzyme. Two ml of the crude mitochondrial pellet were gently homogenised to resuspend any particulate material and loaded onto a

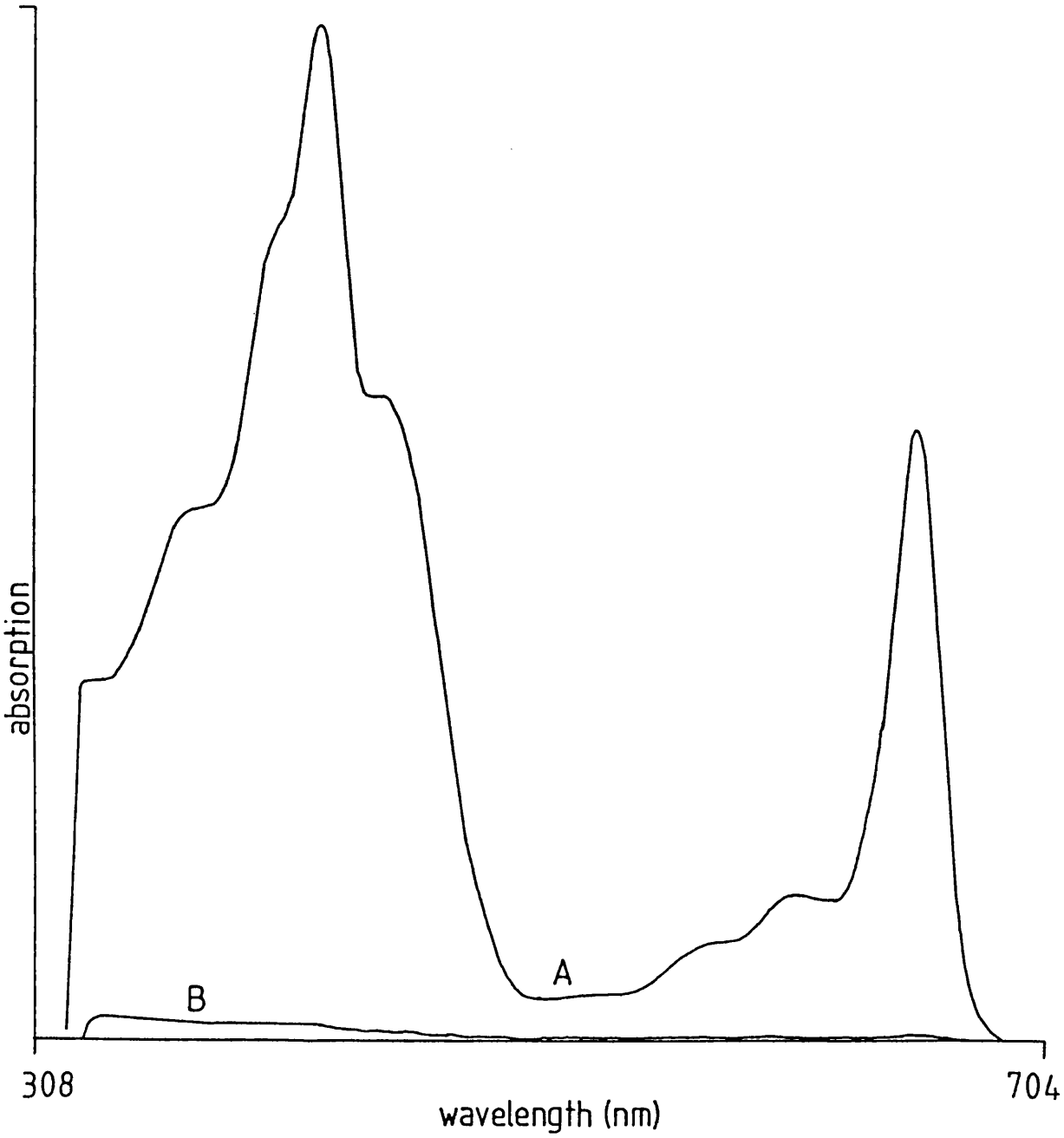


Fig.44 Chlorophyll and carotenoid contamination of crude and Percoll purified mitochondria.

Continuous spectrophotometric scan between 704 and 308nm of acetone extracts from (A) crude mitochondrial pellet and (B) Percoll purified mitochondria. Four mg of protein were extracted in both cases and the scan illustrates the cleanliness of Percoll purified mitochondria with respect to chlorophyll and carotenoid contamination. The scan speed was 120nm/min and the scale for both scans was 0-2.0 absorption units.

13.5/28/55% Percoll gradient. The gradient was spun as described and fractionated into thirty four 1-ml fractions. The fractions were assayed for the above enzymes. Table 4 describes the balance sheet for the differential centrifugation and Fig.45 illustrates the gradient profile.

A similar extraction was performed on 200g of red fruit. When the tube was removed from the centrifuge it became obvious that there had been very little separation within the gradient, as no mitochondria could be detected at the interface between the two discontinuities. The resuspended crude mitochondrial pellet contained pectinacious material that bound cellular organelles in a colloidal matrix and had migrated partially into the gradient as a whole. Spinning at a much higher centrifugal force increased separation but still left several peaks of citrate synthase activity throughout the gradient. Attempts were made to remove pectin by altering the speed of the initial spin to 8,000 x g_{max} but the gradient profile was still poor (Fig.46, Table 5). Commercial pectinase could not be used to digest the pectin as the pH at which the enzyme functions (approx. 4.0) would undoubtedly have deleterious effects on many organelles. Other methods employed included the removal of pectin by some form of ion-exchange resin, this was rejected, however, because of the problem of maintaining organelle integrity while removing the pectin.

It was decided that the pectin problem could be overcome in two ways. 1. Mitochondria could be extracted from the mature green fruit up to the Breaker stage. Since it is known that changes in organic acid metabolism occur before cellular degradation begins

TABLE 4

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.02	0.08	0.002	0.24	1.20	
Malate Dehydrogenase	1.60	2.40	1.10	5.30	27.30	
NAD-linked Isocitrate Dehydrogenase				N.D.	0.30	
NAD-linked Malic Enzyme				N.D.	1.10	
Volume (ml)	630.00	20.00	620.00	12.00	6.00	
Total Activity µmoles						
Citrate Synthase	14.80	1.70	1.36	2.94	7.00	88
Malate Dehydrogenase	1063.50	48.20	659.20	63.60	164.00	88
NAD-linked Isocitrate Dehydrogenase					1.60	
NAD-linked Malic Enzyme					6.70	

Enzyme Recoveries from the Differential Centrifugation of Mature Green Tomato Fruit cv. Sonatine.

200g of pericarp were extracted in 600ml of buffer as described.
N.D. not determined.

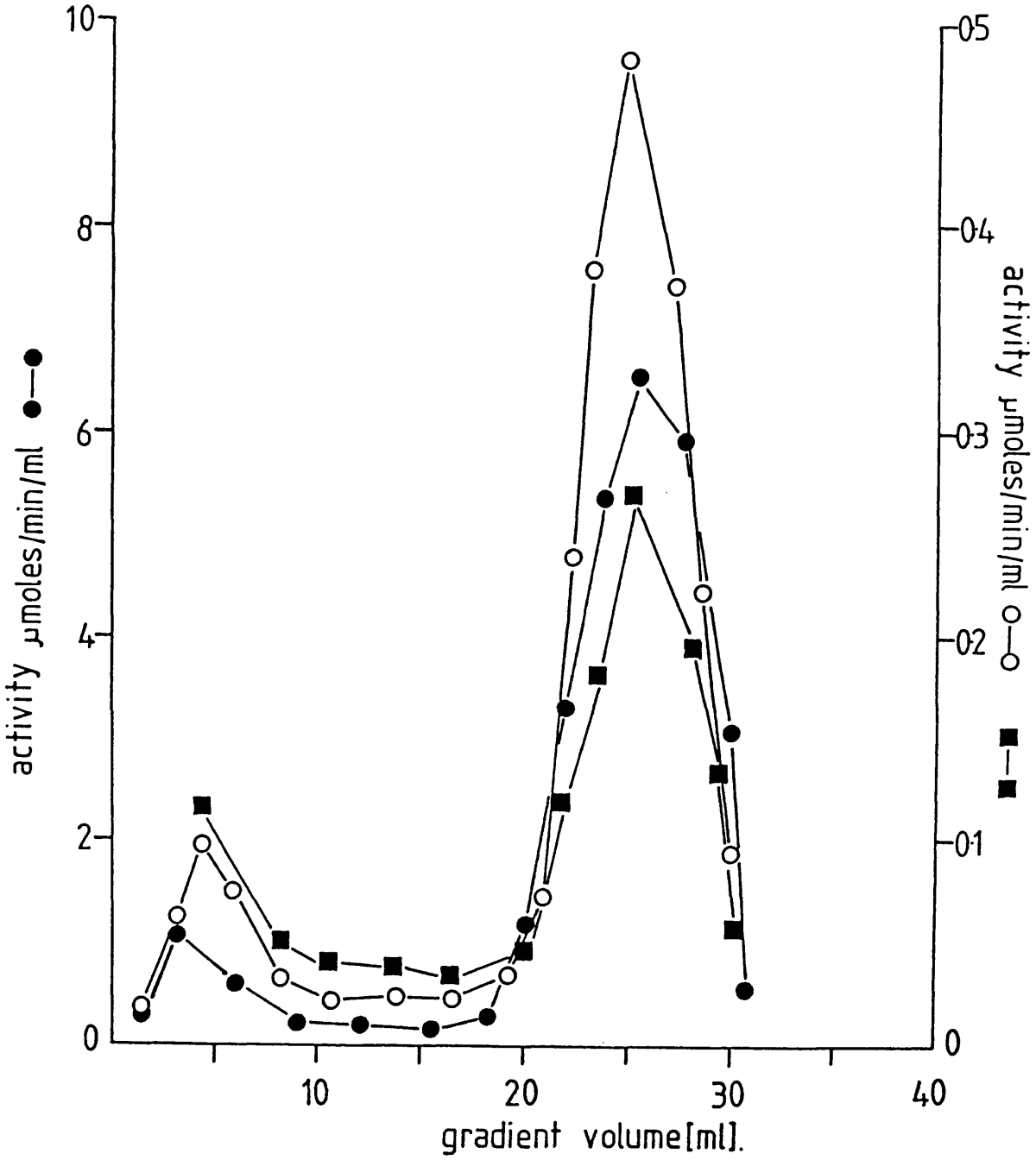


Fig.45 Sub-cellular fractionation of mature green Sonatine.

Fractionation on a discontinuous Percoll gradient (13.5/28/55%) of an enriched mitochondrial/microbody pellet extracted from mature green Sonatine. Enzymes are: (○——○), citrate synthase; (●——●), malate dehydrogenase and (■—■), NAD-linked malic enzyme. Recoveries were: citrate synthase 87%, malate dehydrogenase 88% and NAD-linked malic enzyme 92%.

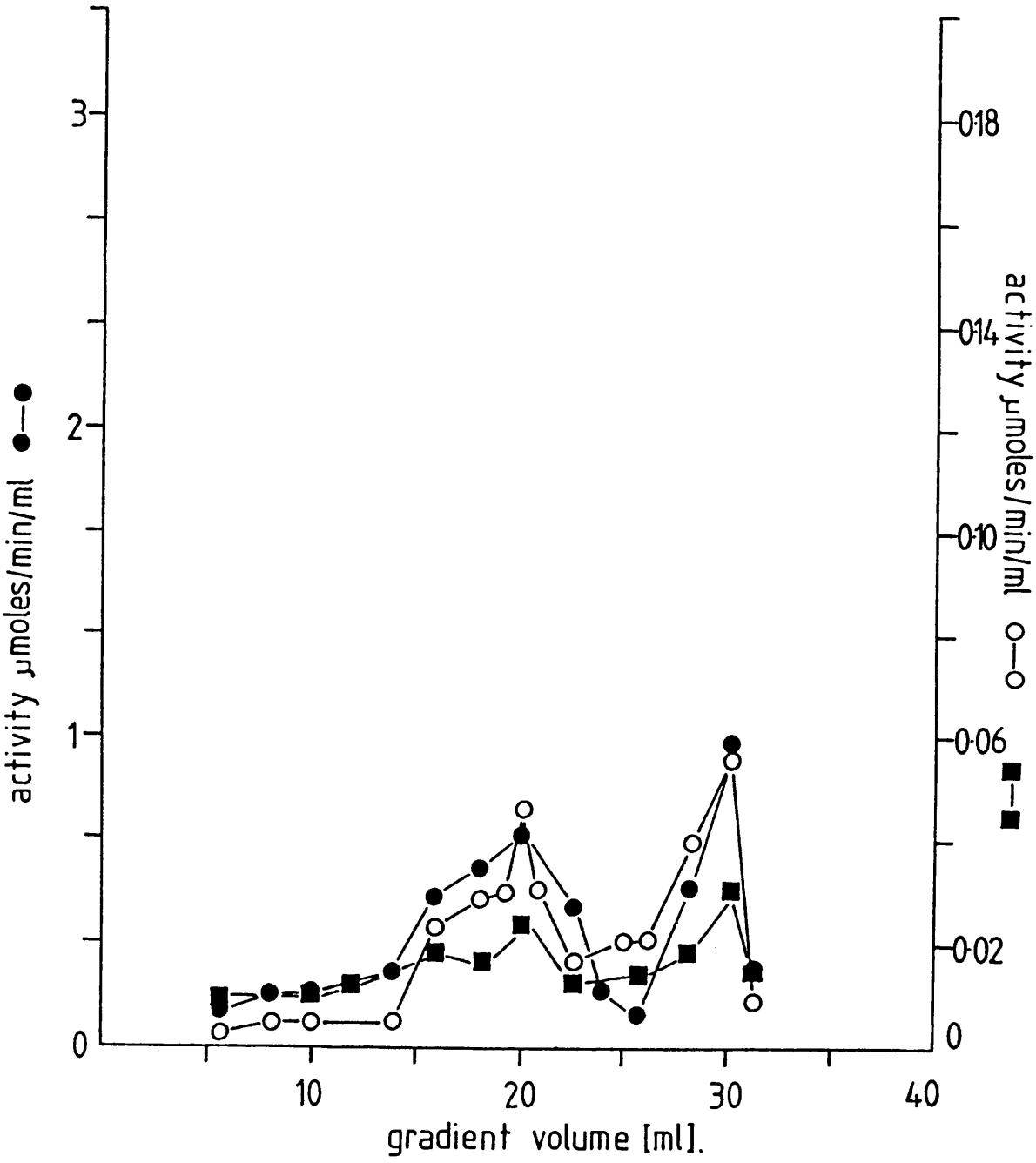


Fig.46 Sub-cellular fractionation of ripe Sonatine.

Fractionation on a discontinuous Percoll gradient (13.5/28/55%) of an enriched mitochondrial/microbody pellet extracted from ripe Sonatine. Marker enzymes are: (○——○), citrate synthase, (●——●), malate dehydrogenase and (■—■), NAD-linked malic enzyme. Recoveries were: citrate synthase 78%, malate dehydrogenase 76% and NAD-linked malic enzyme 86%.

TABLE 5

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.01	0.04	0.002	0.14	0.36	
Malate Dehydrogenase	0.67	2.50	0.48	4.67	6.14	
NAD-linked Isocitrate Dehydrogenase				N.D.	0.04	
NAD-linked Malic Enzyme				N.D.	0.26	
Volume (ml)	590.00	20.00	580.00	5.50	5.50	
Total Activity µmoles						
Citrate Synthase	6.00	0.88	1.27	0.77	2.00	81
Malate Dehydrogenase	398.40	49.80	283.40	25.70	33.70	98.6
NAD-linked Isocitrate Dehydrogenase					0.21	
NAD-linked Malic Enzyme					1.44	

Enzyme Recoveries from the Differential Centrifugation of Ripe Tomato Fruit cv. Sonatine.

200g of pericarp were extracted in 600ml of buffer as described.
N.D. not determined.

(i.e. before the appearance of pectin), this should provide enough data on the changes in specific activity of the mitochondrial enzymes during ripening. 2. Using the modified gas atmosphere storage system would allow a more controlled experiment over a longer time period, while at the same time removing the pectin problem completely. This experiment had the added advantage of helping to determine whether, after the initial fall in specific activity, the residual activity remained constant when the enzymes were extracted from mitochondria.

Analysis of Enzyme Specific Activity from Purified Mitochondria Extracted from Ripening Tomato Fruit cv. Sarina.

At the time of starting the first experiment, it was discovered that the nursery supplying Sonatine had switched production to a new cultivar, Marathon. Sonatine were no longer grown in this country and the nearest equivalent was a cultivar named Sarina that was grown by a single nursery at Wimbourne. It was decided to use this cultivar for the first experiment and Marathon for the second experiment. This would indicate whether the phenomenon was general or cultivar specific.

Fruit were purchased at the mature green stage and allowed to stand at ambient temperature to ripen. Three separate extractions were performed and by the last extraction the fruit had just reached the Breaker stage. It must be stressed, however, that these fruit did not ripen synchronously, which meant that the experiment lasted 2 weeks, rather than the expected 7 or 8 days. Tables 6 - 9 show the balance sheet for the differential centrifugation and the

TABLE 6

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.028	0.20	0.008	0.11	1.00	
Malate Dehydrogenase	1.85	4.42	1.53	1.77	24.90	
NAD-linked Isocitrate Dehydrogenase				N.D.	0.24	
NAD-linked Malic Enzyme				N.D.	3.01	
Volume (ml)	600.00	22.00	590.00	13.5	5.20	
Total Activity µmoles						
Citrate Synthase	16.72	4.52	1.73	2.48	5.16	83
Malate Dehydrogenase	1109.31	97.20	901.14	23.80	129.64	103.8
NAD-linked Isocitrate Dehydrogenase					1.25	
NAD-linked Malic Enzyme					15.70	

Enzyme Recoveries from the Differential Centrifugation of Mature Green Tomato Fruit c.v. Sarina.

200g of pericarp were extracted in 600ml of buffer as described.
N.D. not determined.

TABLE 7

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.26	0.132	0.004	0.160	0.734	
Malate Dehydrogenase	1.57	2.57	1.20	2.41	18.10	
NAD-linked Isocitrate Dehydrogenase				N.D.	0.22	
NAD-linked Malic Enzyme				N.D.	2.23	
Volume (ml)	580.00	25.00	565.00	14.50	6.40	
Total Activity µmoles						
Citrate Synthase	15.40	3.30	2.50	2.30	4.71	83.4
Malate Dehydrogenase	913.82	64.34	681.27	34.96	115.75	98
NAD-linked Isocitrate Dehydrogenase					1.43	
NAD-linked Malic Enzyme					14.29	

Enzyme Recoveries from the Differential Centrifugation of Breaker Tomato Fruit cv. Sarina.

200g of pericarp were extracted in 600ml of buffer as described.
N.D. not determined.

TABLE 8

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.021	0.437	0.003	0.128	0.404	
Malate Dehydrogenase	1.16	5.38	0.96	2.33	5.78	
NAD-linked Isocitrate Dehydrogenase				N.D.	0.133	
NAD-linked Malic Enzyme				N.D.	1.17	
Volume (ml)	470.00	19.00	450.00	13.00	5.2	
Total Activity µmoles						
Citrate Synthase	9.67	3.79	1.32	1.67	2.10	92
Malate Dehydrogenase	547.82	102.33	434.08	30.30	30.10	108
NAD-linked Isocitrate Dehydrogenase					0.69	
NAD-linked Malic Enzyme					6.12	

Enzyme Recoveries from the Differential Centrifugation of Orange/Green Tomato Fruit c.v. Sarina.

200g of pericarp were extracted in 600ml of buffer as described.
N.D. not determined.

TABLE 9

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.014	0.091	0.001	0.128	0.386	
Malate Dehydrogenase	0.88	2.17	0.66	2.09	4.18	
NAD-linked Isocitrate Dehydrogenase				N.D.	0.104	
NAD-linked Malic Enzyme				N.D.	1.15	
Volume (ml)	465.00	25.00	450.00	11.30	5.40	
Total Activity µmoles						
Citrate Synthase	6.49	2.29	0.73	1.47	2.10	101
Malate Dehydrogenase	411.17	54.26	29.80	23.63	22.57	97
NAD-linked Isocitrate Dehydrogenase					0.56	
NAD-linked Malio Enzyme					6.23	

Enzyme Recoveries from the Differential Centrifugation of Orange Tomato Fruit cv. Sarina.

200g of pericarp were extracted in 600ml of buffer as described.
N.D. not determined.

percentage recovery for each enzyme. Tables 10 and 11 illustrate the distribution of cytocolic and mitochondrial MDH during the experiment. Figs. 47 - 50 illustrate the gradient profiles for each of the 3 days. It is apparent from the data that, with the exception of NAD-linked isocitrate dehydrogenase, all the enzymes fell to approximately 35% of their initial values. In addition, the ratio of mitochondrial to cytosolic MDH remained relatively constant throughout and, although not shown, the amount of protein recovered in the mitochondrial peak was between a quarter and a half of that loaded onto the gradient.

The Specific Activity of Citric Acid Cycle Enzymes from the Mitochondria of Tomato Fruit cv. Marathon Stored in a Modified Gas Atmosphere

The protocol for this experiment was identical to the initial storage experiment with the exception that mitochondria were extracted rather than present in a crude homogenate. The experiment was concluded after 4 weeks and it was apparent that the phenomenon seen in Sonatine and Sarina, also occurred in Marathon. The specific activities of citrate synthase and MDH fell by 45% during the first week and then by a further 5% during the following 2 weeks. The gradient profiles are illustrated in Figs. 51 and 52. Enzyme recoveries from the differential centrifugation are shown in Tables 12 - 14 and the distribution of MDH in Tables 15 and 16.

TABLE 10

DAY	TOTAL MALATE DEHYDROGENASE	CYTOSOLIC MALATE DEHYDROGENASE	MITOCHONDRIAL MALATE DEHYDROGENASE	CYTOSOLIC / TOTAL %	MITOCHONDRIAL / TOTAL %
1	1109.30	901.12	153.37	81.2	13.8
2	913.82	675.24	150.64	73.8	16.4
3	547.74	434.08	60.28	79.2	11.0
4	411.09	298.39	45.98	72.5	11.2

The Distribution of Cytosolic and Mitochondrial Malate Dehydrogenase in Ripening Tomato Fruit cv. Sarina.

Figures are expressed as total activity (μmoles per fraction). The two final columns do not sum to 100% because 8-10% of the total activity always sedimented with the first spin.

Day 1 is equivalent to mature green fruit.

Day 2 is equivalent to breaker fruit.

Day 3 is equivalent to orange/green fruit.

Day 4 is equivalent to orange fruit.

TABLE 11

DAY	CYTOSOLIC MALATE DEHYDROGENASE	MITOCHONDRIAL MALATE DEHYDROGENASE
1	100	100
2	75	98
3	48	40
4	33	30

The Change in Activity of Cytosolic and Mitochondrial Malate Dehydrogenase Extracted from Ripening Tomato Fruit cv. Sarina between the Mature Green and Orange Stages.

Figures are expressed as percentages of the Day 1 activities for both fractions.

Day 1 is equivalent to mature green fruit.

Day 2 is equivalent to breaker fruit.

Day 3 is equivalent to orange/green fruit.

Day 4 is equivalent to orange fruit.

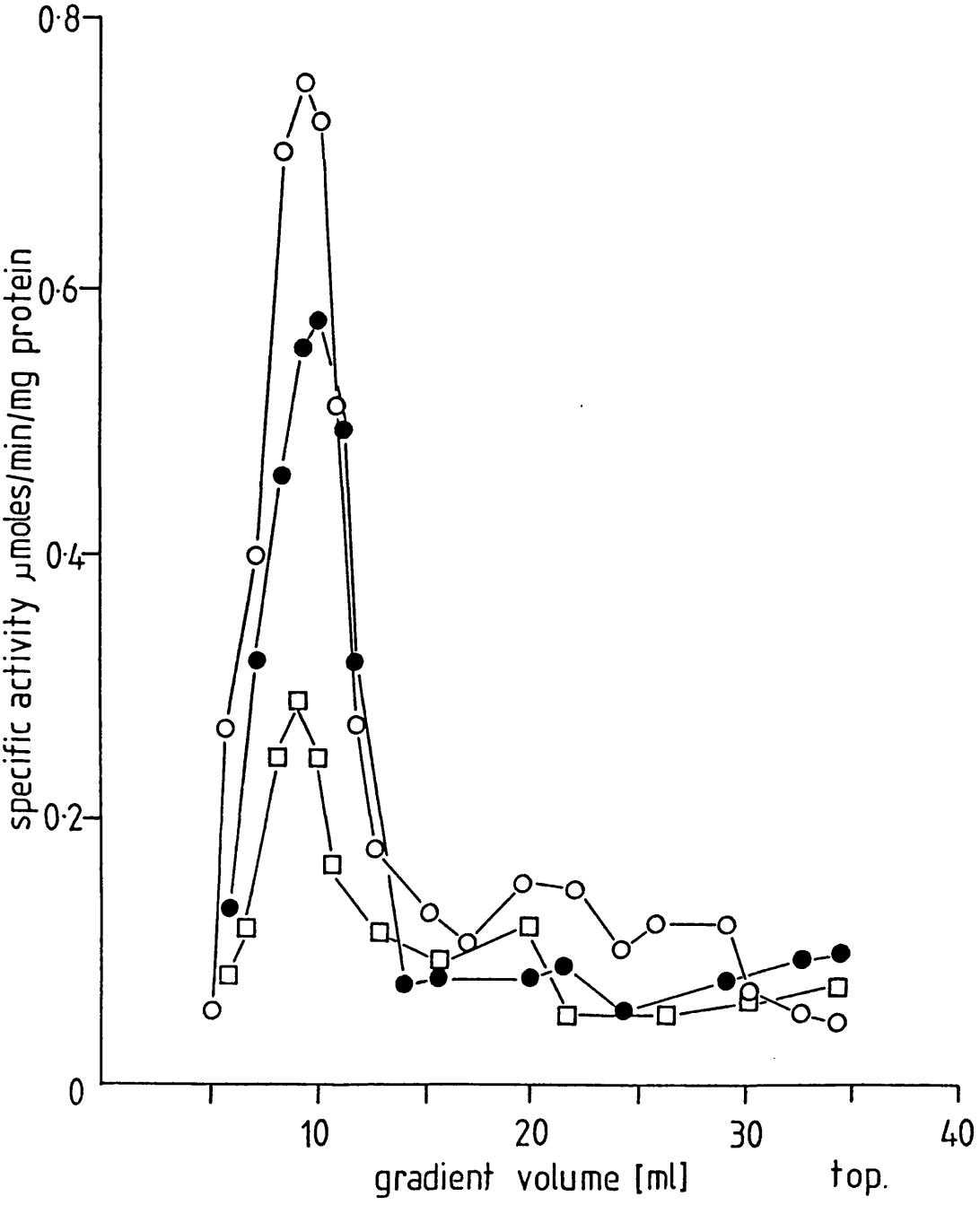


Fig.47 Changes in specific activity of citrate synthase extracted from the purified mitochondria of ripening tomato fruit cv. Sarina.

Specific activity of citrate synthase from a discontinuous Percoll gradient (13.5/28/55%) loaded with an enriched crude mitochondrial/microbody fraction from: (O——O), mature green; (●——●), breaker and (□—□), orange/green, Sarina tomato fruit. Recoveries were: mature green 108%. breaker 101% and orange/green 86%. Protein loaded on each gradient in 3ml was as follows: mature green, 15.4mg; breaker, 12.1mg and orange/green, 6.78mg.

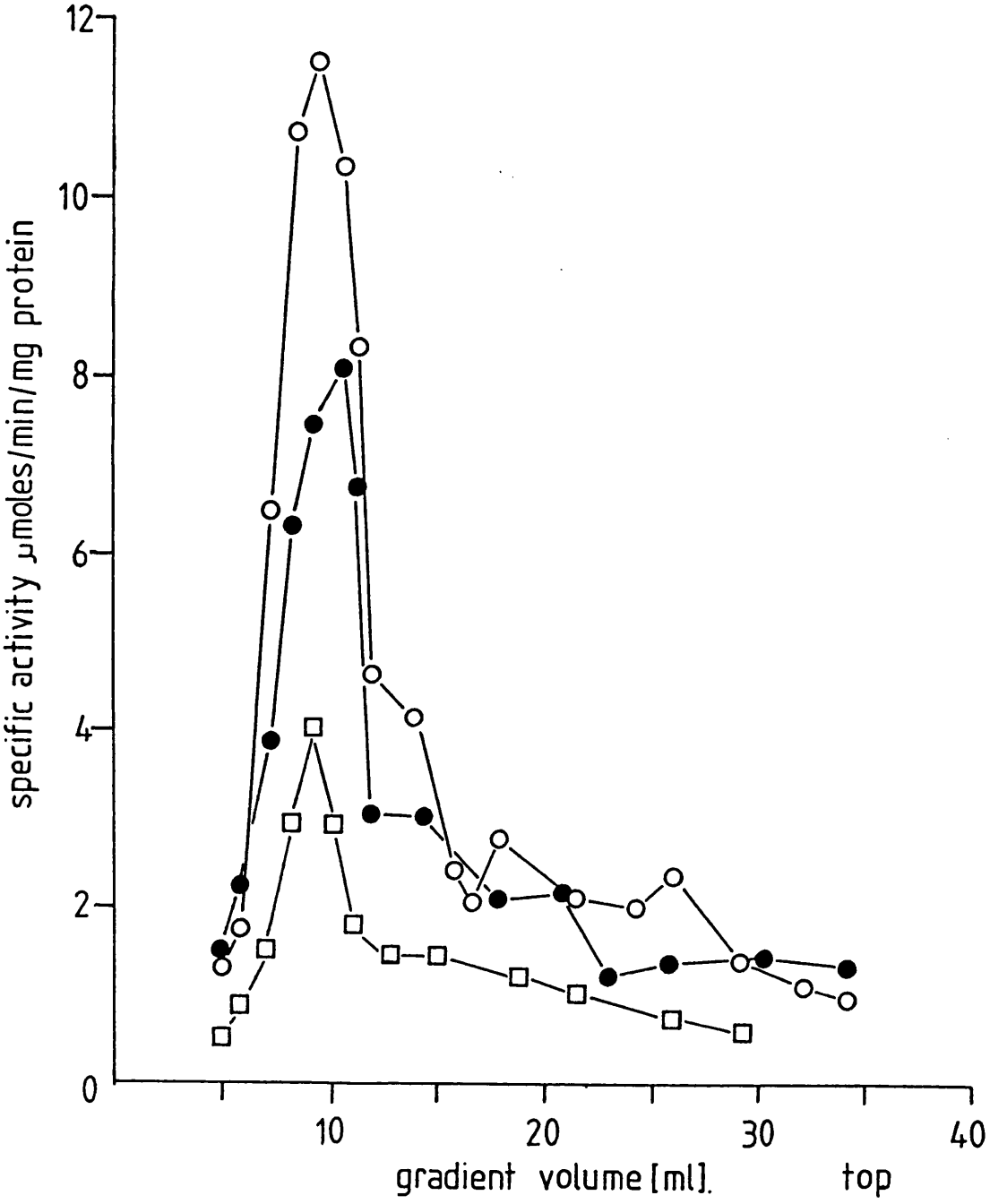


Fig.48 Changes in specific activity of malate dehydrogenase extracted from the purified mitochondria of ripening tomato fruit cv. Sarina.

Specific activity of malate dehydrogenase from a discontinuous Percoll gradient (13.5/28/55%), loaded with an enriched mitochondrial/microbody fraction from: (O——O), mature green; (●——●), breaker and (□—□), orange/green, Sarina tomato fruit. Recoveries were as follows: mature green 88%, breaker 85% and orange/green 103%. Protein loaded on each gradient in 3ml was as follows: mature green 15.4mg, breaker 12.1mg and orange/green 6.78mg.

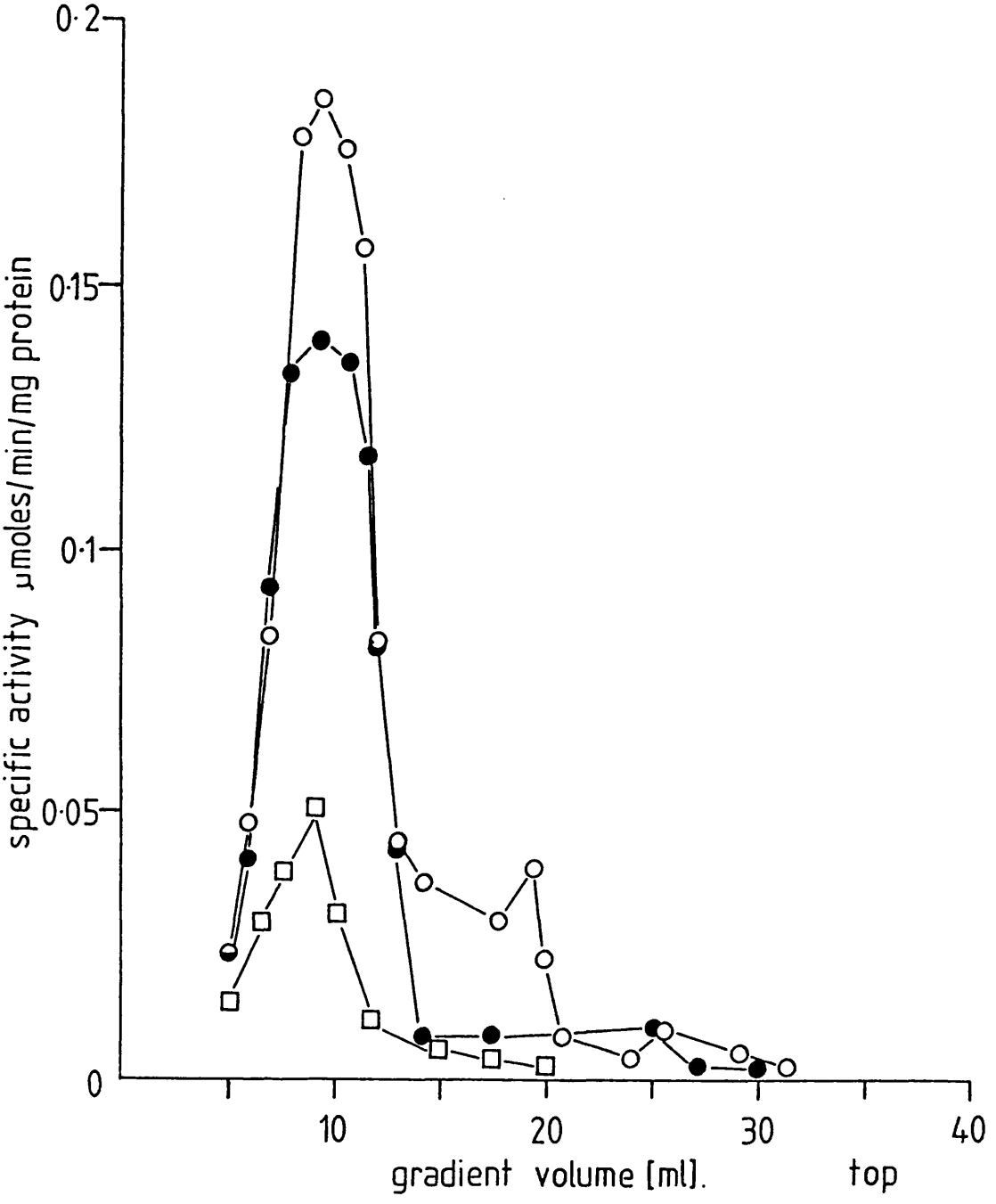


Fig.49 Changes in specific activity of NAD-linked isocitrate dehydrogenase extracted from the purified mitochondria of ripening tomato fruit cv. Sarina.

Specific activity of NAD-linked isocitrate dehydrogenase from a discontinuous Percoll gradient (13.5/28/55%), loaded with a crude enriched mitochondrial/microbody fraction from: (O——O), mature green; (●——●), breaker and (□—□), orange/green Sarina tomato fruit. Reccveries were as follows: mature green 108%, breaker 88% and orange/green 74%. Protein loaded on each gradient was as follows: mature green 15.4mg, breaker 12.1mg and orange/green 6.78mg.

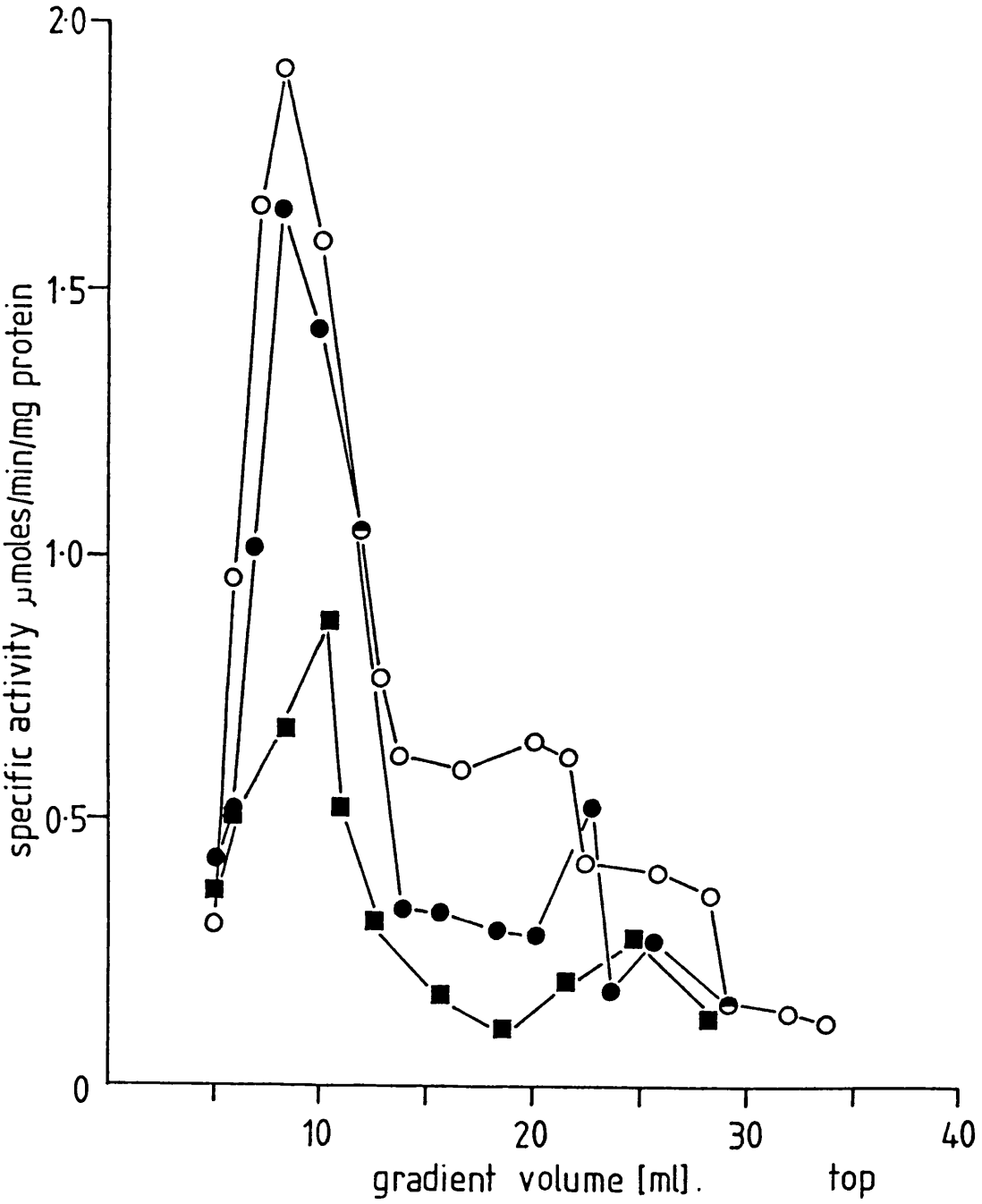


Fig.50 Changes in specific activity of NAD-linked malic enzyme extracted from the purified mitochondria of ripening tomato fruit cv. Sarina.

Specific activity of NAD-linked malic enzyme from a discontinuous Percoll gradient (13.5/28/55%), loaded with a crude enriched mitochondrial/microbody fraction from: (O——O), mature green; (●——●), breaker and (□—□), orange/green, Sarina tomato fruit. Recoveries were as follows: mature green 120%. breaker 125% and orange/green 101%. Protein loaded on each gradient was as follows: mature green 15.4mg, breaker 12.1mg and orange/green 6.78mg.

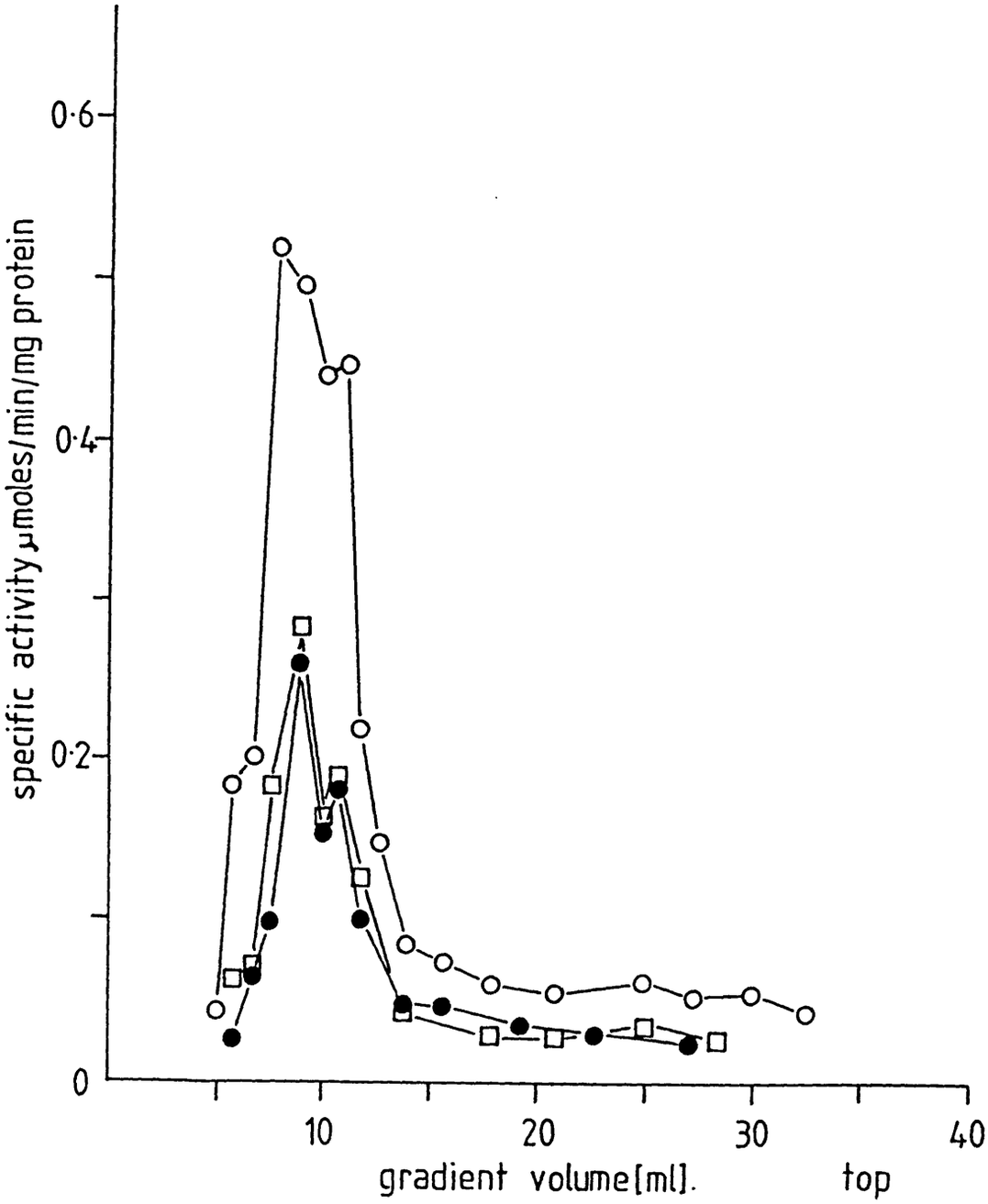


Fig.51 Changes in specific activity of citrate synthase extracted from the purified mitochondria of stored tomato fruit cv. Marathon.

Specific activity of citrate synthase from a discontinucus Percoll gradient (13.5/28/55%) loaded with a crude mitochondrial/microbody fraction from: (O——O), mature green Marathon tomato fruit; (□—□), Marathon stored for one week in an atmosphere of 6% CO_2, 6% O_2 and 88% N_2; (●——●) Marathon stored for three weeks in an atmosphere of 6% CO_2, 6% O_2 and 88% N_2. Recoveries were: mature green 95%, one week in store 86% and three weeks in store 91%.

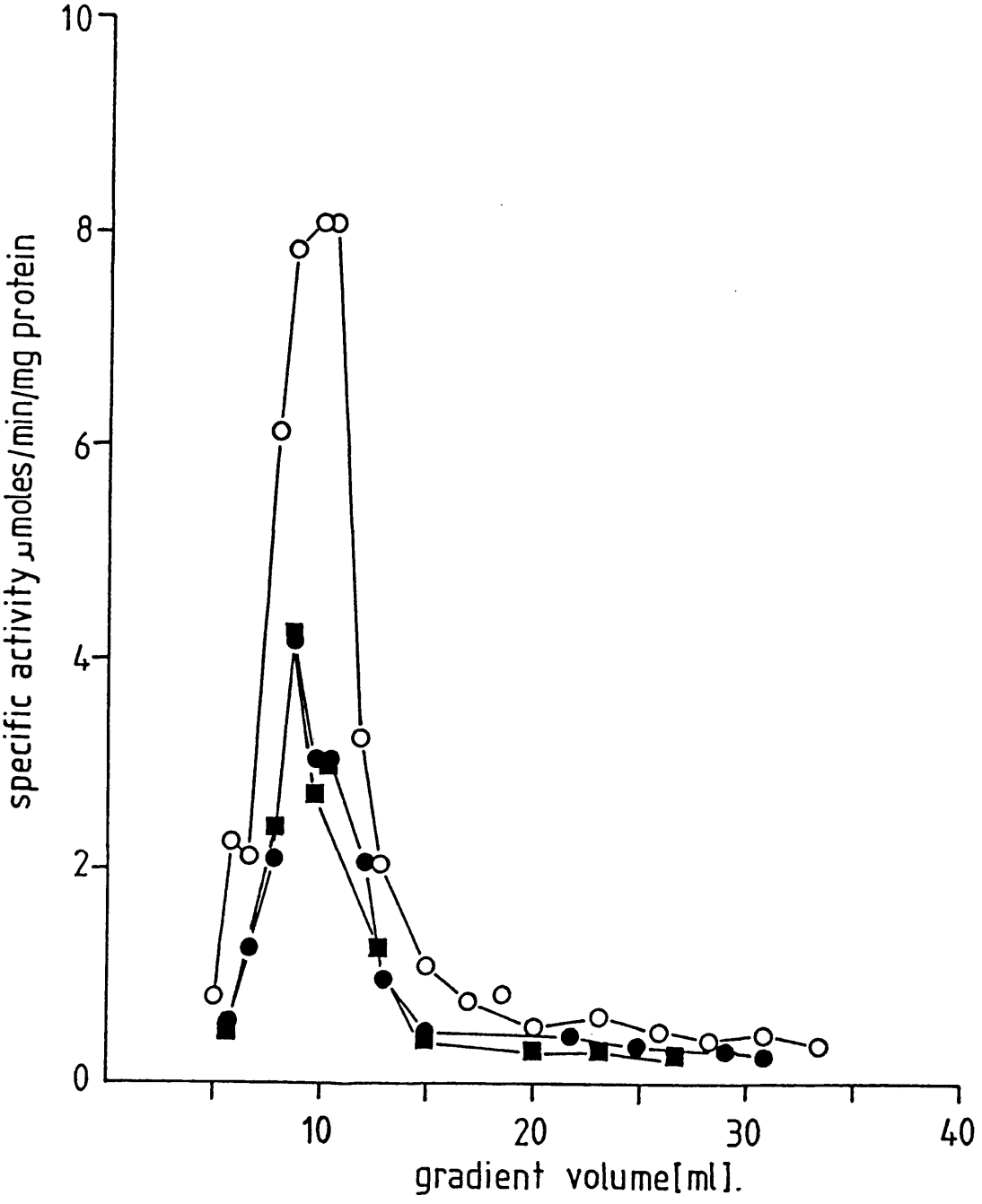


Fig.52 Changes in specific activity of malate dehydrogenase extracted from the purified mitochondria of stored tomato fruit cv. Marathon.

Specific activity of malate dehydrogenase from a discontinuous Percoll gradient (13.5/28/55%) loaded with a crude mitochondrial/microbody fraction from: (O——O), mature green Marathon tomato fruit; (●——●), Marathon stored for one week in an atmosphere of 6% CO_2, 6% O_2 and 88% N_2 and (■—■), Marathon stored for three weeks in an atmosphere of 6% CO_2, 6% O_2 and 88% N_2. Recoveries were: mature green 92%, one week in store 85% and three weeks in store 93%.

TABLE 12

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.029	0.147	0.001	0.477	1.32	
Malate Dehydrogenase	1.92	3.93	1.32	2.83	21.86	
Volume (ml)	640.00	15.50	620.00	13.00	6.20	
Total Activity µmoles						
Citrate Synthase	18.82	2.27	1.17	6.21	8.23	95
Malate Dehydrogenase	1234.72	61.73	813.50	36.97	135.70	85

Enzyme Recoveries from the Differential Centrifugation of Mature Green Tomato Fruit cv. Marathon Stored in 6% CC_2, 6% O_2 and 88% N_2

200g of pericarp were extracted in 600ml of buffer as described.

Control fruit assayed before storing.

TABLE 13

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.020	0.047	0.003	0.245	0.808	
Malate Dehydrogenase	1.41	1.60	1.02	1.45	15.35	
Volume (ml)	642.00	30.00	620.00	12.00	5.00	
Total Activity µmoles						
Citrate Synthase	12.94	1.47	1.76	2.94	4.04	79
Malate Dehydrogenase	903.05	48.23	637.94	17.68	80.38	87

Enzyme Recoveries from the Differential Centrifugation of Mature Green Tomato Fruit cv. Marathon Stored in 6% CO_2, 6% O_2 and 88% N_2

200g of pericarp were extracted in 600ml of buffer as described.

Fruit assayed after one week in store.

TABLE 14

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.020	0.147	0.003	0.402	1.54	
Malate Dehydrogenase	1.36	3.536	0.96	1.67	12.86	
Volume (ml)	628.00	15.00	605.00	8.50	6.30	
Total Activity µmoles						
Citrate Synthase	12.94	2.20	1.69	1.55	4.48	77
Malate Dehydrogenase	858.19	53.05	583.60	14.30	81.02	85

Enzyme Recoveries from the Differential Centrifugation of Mature Green Tomato Fruit cv. Marathon Stored in 6% CO_2, 6% O_2 and 88% N_2

200g of pericarp were extracted in 600ml of buffer as described.

Fruit assayed after three weeks in store.

TABLE 15

	TOTAL MALATE DEHYDROGENASE	CYTOSOLIC MALATE DEHYDROGENASE	MITOCHONDRIAL MALATE DEHYDROGENASE	CYTOSOLIC / TOTAL %	MITOCHONDRIAL / TOTAL %
Pre-store (control)	1234.72	813.50	172.66	66	14
Week 1	903.05	637.94	98.07	70	11
Week 3	860.93	583.60	97.10	68	11

The Distribution of Cytosolic and Mitochondrial Malate Dehydrogenase in Ripening Tomato Fruit cv. Marathon Stored in 6% CO_2, 6% O_2 and 88% N_2

Figures are expressed as total activity (μmoles per fraction). The two final columns do not sum to 100% because 8-10% of the total activity always sedimented with the first spin.

TABLE 16

	CYTOSOLIC MALATE DEHYDROGENASE	MITOCHONDRIAL MALATE DEHYDROGENASE
Pre-store (control)	100	100
Week 1	78	60
Week 3	72	60

The Change in Activity of Cytosolic and Mitochondrial Malate Dehydrogenase Extracted from Ripening Tomato Fruit cv. Marathon stored in 6% CO_2, 6% O_2 and 88% N_2

The figures are expressed as percentages of the control activity for both fractions.

Investigation of Changes in the Specific Activity of Citric Acid Cycle Enzymes from the Mitochondria of Immature Tomato Fruit

In an attempt to determine whether the characteristic changes in specific activities of the enzymes under study were a function of ripening or the result of removing the fruit from the vine, the mitochondria from immature Marathon were purified and the respective enzymes assayed.

Immature Marathon (less than 25mm in diameter) were purchased from a local nursery and stored in ambient conditions. Identical weights (165g) were sampled at weekly intervals for 4 weeks. At the end of this period the immature fruit exhibited a complete lack of ripening characteristics; the pericarp was still firm and there was no sign of colour change. Enzyme recoveries from the differential centrifugation are shown in Tables 17 - 19 and the distribution of MDH in Tables 20 and 21.

An equal volume (3ml) of resuspended crude mitochondrial pellet was loaded onto the Percoll gradient during each fractionation. Citrate synthase showed a fall in activity during the first week of 45% and then a subsequent fall of 10% during the following 2 weeks (Fig.53). However, when the protein figures were calculated, the data indicated virtually no difference in specific activity (Fig.54). The experiment was repeated, with the exception that instead of loading a constant volume of the resuspended crude mitochondrial pellet onto the Percoll gradient, a constant activity was applied (40 units). Citrate synthase showed a 30% fall in activity, though the specific activity was hardly altered. During

TABLE 17

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.044	0.211	0.003	0.358	1.54	
Malate Dehydrogenase	2.81	4.50	1.96	6.75	40.19	
Volume (ml)	550.00	22.00	532.00	14.50	6.40	
Total Activity µmoles						
Citrate Synthase	24.26	4.63	1.69	5.19	9.88	88.2
Malate Dehydrogenase	1547.42	99.03	1044.05	97.91	257.23	97.0

Enzyme Recoveries from the Differential Centrifugation of Small (Immature) Tomato Fruit cv. Marathon held in Ambient Conditions.

165g of pericarp were extracted in 500ml of buffer as described.

Control fruit assayed same day as harvested.

TABLE 18

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.020	0.128	0.003	0.275	0.845	
Malate Dehydrogenase	1.76	2.36	1.24	2.93	24.11	
Volume (ml)	572.00	21.00	550.00	15.00	6.40	
Total Activity µmoles						
Citrate Synthase	15.97	2.68	1.61	3.02	5.88	83.0
Malate Dehydrogenase	1011.57	49.63	685.28	44.00	154.34	92.2

Enzyme Recoveries from the Differential Centrifugation of Small (Immature) Tomato Fruit cv. Marathon held in Ambient Conditions.

165g of pericarp were extracted in 500ml of buffer as described.

Fruit assayed after one week in ambient conditions.

TABLE 19

	HOMOGENATE	FIRST SPIN	SUPERNATANT	WASH	MITOCHONDRIAL PELLET	RECOVERY %
Activity µmoles/ml						
Citrate Synthase	0.027	0.110	0.003	0.228	1.00	
Malate Dehydrogenase	1.76	2.02	1.27	3.12	24.11	
Volume (ml)	580.00	21.00	558.00	17.00	6.00	
Total Activity µmoles						
Citrate Synthase	15.78	2.31	1.64	3.87	5.95	87.3
Malate Dehydrogenase	1025.72	42.50	708.71	53.02	144.69	92.5

Enzyme Recoveries from the Differential Centrifugation of Small (Immature) Tomato Fruit cv. Marathon held in Ambient Conditions.

165g of pericarp were extracted in 500ml of buffer as described.

Fruit assayed after three weeks in ambient conditions.

TABLE 20

	TOTAL MALATE DEHYDROGENASE	CYTOSOLIC MALATE DEHYDROGENASE	MITOCHONDRIAL MALATE DEHYDROGENASE	CYTOSOLIC / TOTAL %	MITOCHONDRIAL / TOTAL %
Pre-store (control)	1547.40	1044.10	355.10	67	23
Week 1	1011.60	685.20	198.20	67	20
Week 3	1025.70	708.60	197.71	69	20

The Distribution of Cytosolic and Mitochondrial Malate Dehydrogenase in Immature Tomato Fruit cv. Marathon held in Ambient Conditions

Figures are expressed as total activity (µmoles per fraction). The two final columns do not sum to 100% because 8-10% of the total activity always sedimented with the first spin.

TABLE 21

	CYTOSOLIC MALATE DEHYDROGENASE	MITOCHONDRIAL MALATE DEHYDROGENASE
Pre-store (control)	100	100
Week 1	65	55
Week 2	67	58

The Change in Activity of Cytosolic and Mitochondrial Malate Dehydrogenase Extracted from Immature Tomato Fruit cv. Marathon held in Ambient Conditions.

The figures are expressed as percentages of the control activity for both fractions.

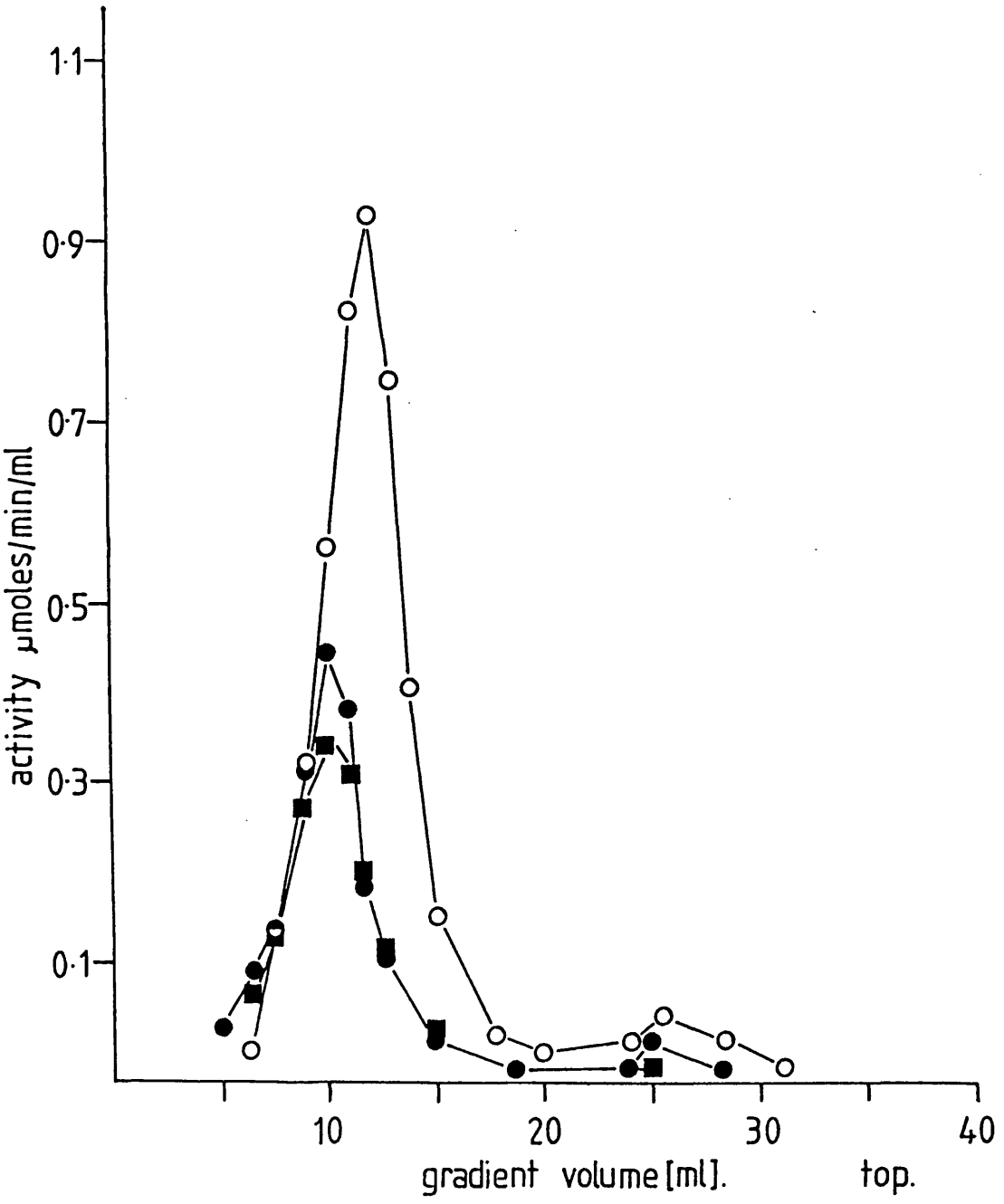


Fig.53 Changes in activity of citrate synthase extracted from the purified mitochondria of immature tomato fruit cv. Marathon.

Activity of citrate synthase from a discontinuous Percoll gradient (13.5/28/55%) loaded with 3ml of a crude enriched mitochondrial/microbody fraction from: (O——O), immature Marathon tomato fruit; (●——●), immature Marathon stored for one week in ambient conditions and (■—■), immature Marathon stored for three weeks in ambient conditions. Recoveries were: immature 104%, one week in ambient conditions 99% and three weeks in ambient conditions 93%.

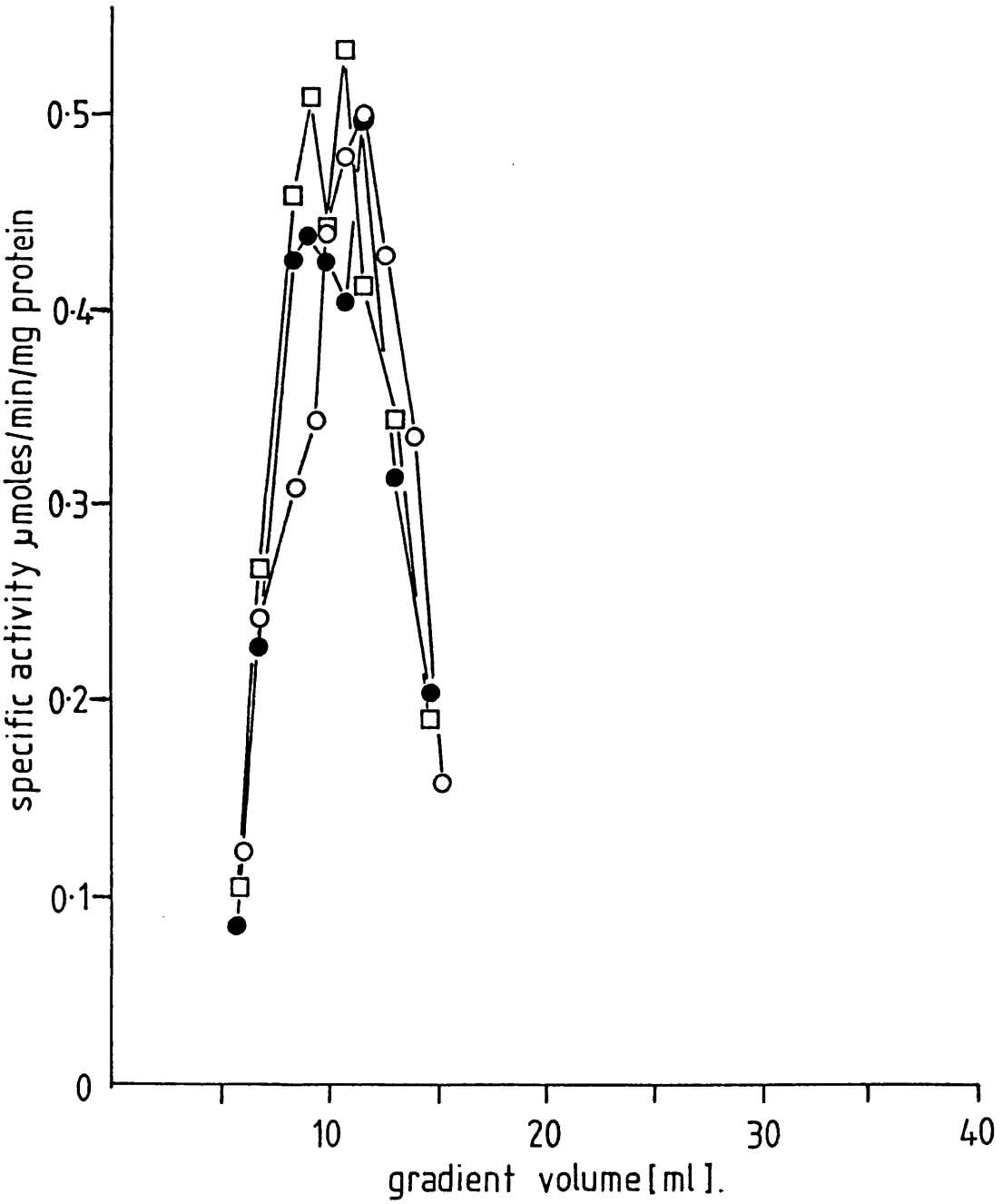


Fig.54 Changes in specific activity of citrate synthase extracted from the purified mitochondria of immature tomato fruit cv. Marathon.

Specific activity of citrate synthase from a discontinuous Percoll gradient (13.5/28/55%) loaded with 3ml of a crude enriched mitochondrial/microbody fraction from: (O——O), immature Marathon tomato fruit; (□—□), immature Marathon stored for one week in ambient conditions and (●——●), immature Marathon stored for three weeks in ambient conditions. Protein measurements were taken for the peak activity fractions only.

the first fractionation, 40 units were contained in a volume of 2.4ml and the total protein was 17.7mg. At the end of the second week, a volume required to load the same amount of protein had risen to 3.4ml. An identical amount of fruit had been extracted in each case and the mitochondrial pellet was resuspended in the same volume (5.8ml).

DISCUSSION

The citric acid cycle was first postulated in 1937 by Krebs and Johnson. The association between the mitochondrion and the citric acid cycle was confirmed twelve years later (Kennedy and Lehninger, 1949) and a further two years elapsed before the same association was established in plant tissue (Millard et al., 1951). Since 1951 the crucial importance of the cycle has been verified, and the functions of the cycle are now known to extend from energy conservation to the control of both anabolic and anaplerotic processes. Animal and microbial sources have been utilised in the vast majority of subsequent research into the control of the cycle. Although investigations of the cycle and its regulation have been undertaken in plants (Wiskich, 1980), it is striking that relatively little is known about those plants, or fruits, that accumulate and rapidly metabolise high concentrations of cycle intermediates.

The control of individual enzymes within the cycle appears to differ between plant and animal sources. Thus NAD-dependent isocitrate dehydrogenase from plants is strongly inhibited by the mole fraction of NADH as a mole fraction of NADH + NAD^+ and unlike the enzyme from animal sources, is unaffected by adenine nucleotides. However, it is in the control of malate oxidation that the main difference occurs between the citric acid cycle of plants and animals. Plant mitochondria possess an NAD-dependent malic enzyme (Macrae, 1971) which enables them to oxidise malate to pyruvate and, therefore, theoretically to oxidise completely any

cycle intermediate without the need to replenish exogenous acetyl-CoA (Palmer, 1979; Palmer and Moller, 1982). It is claimed (Palmer and Moller, 1982) that animal mitochondria do not oxidise malate due to the unfavourable equilibrium constant of malate dehydrogenase, thereby implying that animal mitochondria do not contain an NAD-dependent malic enzyme. However, Sauer et at. (1979) established the presence of malic enzyme that only functioned with NAD^+ as co-factor in mitochondria isolated from human intestinal mucosa, and showed it to be responsible for the oxidative decarboxylation of glutamine derived malate. In plants and plant organs that accumulate large concentrations of malate and citrate, it would be of physiological advantage to be able to oxidise cycle intermediates such as malate, without the need to replenish acetyl-CoA.

Another area of difference between animal and plant mitochondria is in control of the redox state, or $NADH/NAD^+$ ratio. Animal mitochondria maintain the $NADH/NAD^+$ ratio approximately 100 times greater than that of the cytoplasm (Williamson et al., 1972). However, since animal mitochondria cannot oxidise cytoplasmic NADH, complex shuttle systems are involved in transporting reducing equivalents across the mitochondrial membrane. This in contrast to the ease in which external NADH is oxidised in many plant mitochondria (Palmer and Moller, 1982). In addition, there is now evidence which suggests that NAD^+ is taken up by plant mitochondria (Tobin et al., 1980). Taken together, this evidence suggests that the $NADH/NAD^+$ ratio plays a much less important role in the control of the citric acid cycle in plants than in animals. It can be postulated that these apparent distinctions in control of the

citric acid cycle between plants and animals relate to the physiological differences between them and their respective responses to rapid changes in their immediate environment. Unfortunately, there are very few data relating enzyme activities to maximum cycle activity (measured by oxygen uptake) from plant sources as distinct from the many studies conducted on animal tissue where, for example, it is known that the initiation of flight in many insects results in a massive increase in oxygen uptake and turnover rate of the cycle (Newsholme and Start, 1977). Further examples are the sudden and sustained energy requirements of the large carnivores, where the efficient and rigorous control of the cycle is of paramount importance. This situation contrasts with that of the largely 'static' plant, in which rapid and sustained changes in respiratory activity are rare.

There are, however, a few circumstances in which increased respiratory rates are found in plants. These increased rates include the specialised pollinating mechanism of the Aracacea, in which the respiratory activity of mitochondria in the spadices increases, although in this particular case the mitochondria are uncoupled and energy is not conserved as ATP but is dissipated as heat via the cyanide-insensitive pathway (James and Elliot, 1955; Hackett, 1957).

The main rapid and transient increase in respiration, however, is found in climacteric fruit, where the increase in respiration in some fruit, such as the breadfruit from South America, can exceed 170ml CO_2/kg/h; in comparison, the tomato produces 20-30ml CO_2/kg/h. What is the physiological significance of this

burst of carbon dioxide and what is its relationship to other biochemical events associated with ripening? The answer to the first part of the question is that although logical suggestions abound, the definitive answer is still not known. The respiratory burst is used as a convenient marker to define the change between maturity and ripeness in climacteric fruit and is associated with the autocatalytic production of ethylene. In many fruits, such as the banana and avocado cv. choquette, the synthesis of ethylene precedes the respiratory burst (Burg and Burg, 1962), while in the avocado cv. fuerte and tomato cv. Rutgers, ethylene synthesis follows the respiratory burst (Kosiyachinda and Young, 1975; Mizrahi et al., 1975). In the case of apple Cox Orange Pippin and the musk melon Cantaloupe, both peaks coincide (Rhodes and Reid, 1975; Lyons et al., 1962). It can be deduced from this that ethylene cannot be the only factor involved in the regulation of the respiratory burst.

The banana is a fruit that has been extensively investigated biochemically. During the respiratory burst, large quantities of starch (20-30% of the fresh weight) are hydrolysed and simple sugars increase from 1 to 14-15%. Young et al. (1975) assayed glycolytic intermediates and enzymes at seven stages during the respiratory climacteric. Their data indicated that fructose 6-phosphate and glucose 6-phosphate were high in green fruit and changed little during ripening. However, fructose 1, 6-diphosphate increased 9-fold during the early climacteric and 20-fold at the peak. A cross-over plot revealed a large cross-over value between fructose 6-phosphate and fructose 1, 6-diphosphate, indicating a regulatory site at phosphofructokinase.

In this study it has been impossible to measure respiratory rates during the whole fruit experiments as the results would have been meaningless in the atmosphere in which the fruit were stored. However, since the rise in ethylene concentration was monitored and it is known that this rise closely follows the respiratory rise, it is reasonable to discuss the two together.

Data from both the storage and ethylene-supplemented experiments show conclusively that major changes in specific activity of several citric acid cycle and related enzymes occurred before ethylene could be detected. The presence of ethylene made no significant difference to the overall change in specific activities of citrate synthase and malate dehydrogenase, and similar changes in the specific activity of these enzymes were observed if fruit were allowed to ripen naturally on the vine. This finding is significant, since it strengthens the argument that ripening of climacteric fruit is not a series of interdependent processes occurring simultaneously, initiated by the onset of ethylene synthesis.

It is possible to speculate that the source of respiratory CO_2 is malate, since the specific activity of NADP-dependent malic enzyme increased by 400% during this period. The 'malate' effect is well documented for malus species (Drouet and Hartmann, 1977). The fall in the specific activity of citrate synthase and malate dehydrogenase coincides with the maximum increase in glucose and fructose concentration, suggesting that neither are major glycolytic substrates at this time.

In complete contrast to the foregoing, are those aspects of ripening such as the appearance of lycopene, polygalacturonase and the increase in invertase activity, which are dependent on ethylene for initiation and continuance of response. There is a very low correlation between acid invertase activity and monosaccharide concentration and it is evident that this enzyme does not contribute to glucose and fructose production during starch hydrolysis; however, it may be required for the hydrolysis of cell wall linkages since it increases in activity concurrently with polygalacturonase. The loss of chlorophyll occurs slowly when fruit are removed from the vine and is apparently not dependent on the presence of ethylene, although the presence of the gas appears to enhance the rate of loss.

The data from the whole fruit experiments indicate that three categories of biochemical changes can be formulated:

1. Changes in the specific activities of citrate synthase, malate dehydrogenase and NADP-dependent malic enzyme with concomitant changes in citrate and malate metabolism, and metabolism of starch to equal quantities of glucose and fructose. These changes occur immediately the fruit are removed from the vine at the mature green stage. They are not initiated or stimulated by ethylene and they do not rely on cell wall breakdown by polygalacturonase.
2. Loss of chlorophyll, which occurs slowly when fruit are removed from the vine but without the changes mentioned in category 3. This process is enhanced by ethylene.

3. The synthesis of lycopene and polygalacturonase, coupled with the increase in specific activity of acid invertase, all of which appear to depend on ethylene for initiation and continuation of response.

The biochemical changes described above for mature green Sonatine, occur to a similar or lesser extent in the cultivars Sarina and Marathon. When the same criteria are applied to the rin mutant, however, distinct differences in organic acid metabolism become evident. The most obvious difference is the continued increase in specific activity of citrate synthase from the mature to the ripe state and the modest fall in specific activity of malate dehydrogenase compared to normal fruit during the same period. Although only two determinations were made for NADP-dependent malic enzyme, the results were identical and showed no change in specific activity between the mature and ripe state but a 40% increase between the immature and ripe states. Despite the large differences in organic acid metabolism shown by the normal and rin fruit, Sisler (1982) has demonstrated that the tissue incorporation of ethylene is very similar in both. It is therefore probable that ethylene binds equally well to its receptor in normal and rin fruit, but that a subsequent step in ethylene metabolism is blocked in the mutant. Alternatively, it may mean that the measured tissue incorporation is of no significance to the mode of action of ethylene. In terms of the respiratory climacteric and ethylene autocatalysis, the rin has been classified as a non-climacteric fruit (Tigchelaar et el., 1978). It is interesting, therefore, that the trend in specific activity of citrate synthase is similar

to that found in the juice vesicles of the non-climacteric fruit satsuma mandarin and sweet lime, although these showed a rise in malate dehydrogenase activity (Hirai and Ueno, 1977). Little change occurred in the activity of NADP-dependent malic enzyme throughout the ripening period. However, during the period of citric acid increase, there was a doubling in the activity of NADP-dependent malic enzyme, while at the same time the activities of citrate synthase and malate dehydrogenase did not change. Although citrate synthase, malate dehydrogenase and NAD-dependent isocitrate dehydrogenase activities increased 6-fold, 9-fold and 5-fold respectively between mid-August and mid-October, there was no increase in respiratory activity; indeed, from the beginning of October the respiratory activity declined. The rin results and the data from mandarin and sweet lime emphasise the difference between climacteric and non-climacteric fruit, but they also give added impetus to the argument that CO_2 evolution via NADP-dependent malic enzyme may be common to both types of fruit, as the period of increase in NADP-dependent malic enzyme in satsuma and sweet lime coincided with the (albeit small) increase in respiration.

Pratt and Workman (1961) were able to advance the start and increase the concentration of CO_2 evolved during the climacteric in tomato fruit cv. Improved Pearson. Their experimental treatment involved passing ethylene at 1000ppm over stored fruit for seven days. The concentration of ethylene used was completely non-physiological, and it is now known that tomato fruit metabolise ethylene (Blomstrom and Beyer, 1980). Apart from ethylene oxide and ethylene glycol, the main oxidation product of ethylene metabolism is CO_2 (Beyer, 1981).

Citrate Synthase and Malate Dehydrogenase

The thermal denaturation studies on purified citrate synthase suggested that only one enzymic form was located in the cell. Although not conclusive, the lack of a biphasic denaturation curve is strong evidence that isoenzymes are not present. It is most unlikely that multiple forms were present in the crude extract, as only one peak of citrate synthase activity was detected at each stage of purification. Supporting evidence for a single enzymic form comes from Zehler et al. (1984) who have shown that the glyoxysomal and mitochondrial isoenzymes of citrate synthase from Ricinus communis have different inactivation temperatures. The mitochondrial enzyme lost half its activity within 10min at 45°C, whereas the glyoxysomal form was completely stable at 47°C.

Citrate synthase from L. esculentum closely resembles in its physical and chemical characteristics, the enzyme from several other eukaryotic sources. It is a 'small' enzyme, almost certainly dimeric in structure, the sub-units being of equal size. In common with many other plant and several bacterial citrate synthases it is inactivated by DTNB. Although the concentration of DTNB used could be considered high (5mM), the same concentration had no effect on the pig heart enzyme. The inactivation was slow for a considerable time and then, within a short period, almost total inactivation occurred. This suggests slow oxidation of accessible thiol groups giving a conformational change, which in turn exposes, and permits the oxidation of, an essential thiol group. The fact that the pig heart enzyme is not susceptible to DTNB inactivation presumably means that in the normal conformational state there are less exposed

inessential thiol groups.

In agreement with Brock and Fletcher (1969), no activation of citrate synthase by indole acetic acid was detected. This is hardly surprising since, as Srere (1972) has pointed out, if direct activation is implied rather than some form of cascade system, then the problem of the intracellular ratio of hormone to protein must be overcome. At any given time, the concentration of citrate synthase would probably be a minimum of 100 times that of indole acetic acid. Although Sarkissian (1970) claimed that indole acetic acid actually bound to citrate synthase, increasing its size, no data on this size change were given.

NADH inhibition was not found with the purified citrate synthase, although in crude extracts inhibition was noticeable, this was almost certainly due to the presence of malate dehydrogenase which leads to a loss of oxaloacetate, one of the substrates of the citrate synthase reaction. In contrast to the findings of Sakamoto et al. (1970), using castor bean tissue, no inhibition of citrate synthase by citrate or malate was detected. The preparations used by Sakamoto et al. (1970) were not pure and the concentrations of citrate and malate giving 50% inhibition were extremely high; the Ki for citrate was 20mM and that for malate was 84mM. The Km values of the enzyme for acetyl-CoA and oxaloacetate are 30μm and 8μm respectively, any inhibition by citrate or malate therefore, would almost certainly be non-physiological.

Virtually no protection against thermal denaturation was afforded by either citrate or isocitrate; however, the protection

afforded by the substrate oxaloacetate was almost total at low concentration. Srere (1972) proposed that the binding of oxaloacetate to the enzyme induces a conformational change which increases the stability of the enzyme and aids proton abstraction from acetyl-CoA. Srere also examined the stability of the binary complex of pig heart citrate synthase with oxaloacetate and found it stable in 4M urea.

The characterisation of malate dehydrogenase began when it became obvious that this enzyme was a major contaminant in the purification of citrate synthase. Two peaks of malate dehydrogenase activity eluted from Gel Red A, one of which was associated with citrate synthase and it was possible that one or more isoenzyme of malate dehydrogenase co-purified with citrate synthase. The investigation of malate dehydrogenase isoenzyme pattern was attempted with the aim of detecting any differences between the mature green and red fruit that would explain the fall in specific activity. Isoenzymes are defined as multiple forms of a given enzyme occurring either in a single individual or in different members of the same species. Isoenzymes can arise in three ways (Harris and Hopkinson, 1976).

1. Through multiple gene loci coding for distinct polypeptide chains of the enzyme.
2. Through multiple allelism at a single locus determining structurally distinct versions of a particular polypeptide chain.
3. Through post-translational modification of enzyme structure.

Isoenzymes of malate dehydrogenase, like those of lactate and alcohol dehydrogenase, have arisen through point mutation of multiple gene copies. The detection of 4 isoenzymes of malate dehydrogenase from L. esculentum cv. Sonatine is in agreement with the work of Hobson (1974), who found 4 isoenzymes in the cultivar Amberlay Cross; one isoenzyme was the major staining band and there were 3 closely-grouped minor staining bands. The Gel Red A elution profile and the isoelectric focussing studies on Sonatine suggest that the most acidic isoenzyme of malate dehydrogenase may be associated with citrate synthase. However, it is anomalous that the elution profile from Gel Red A only indicated 2 peaks of malate dehydrogenase activity, when there were obviously 4 isoenzymes. Additionally, at least a small proportion of the isoenzyme that co-eluted with citrate synthase must have been eluted with the main peak of malate dehydrogenase activity. This may reflect the fact that Matrex gels do not function as true ion-exchangers. There are two pieces of evidence, however, which suggest that the mitochondrial isoenzyme possesses properties that distinguish it from the cytosolic isoenzymes:

1. The isoenzyme that co-eluted with citrate synthase from Gel Red A bound to a thiol-disulphide exchange column with citrate synthase and both enzymes were eluted with 30mM dithiothreitol.
2. The mitochondrial isoenzyme can be separated from the cytosolic forms by Percoll fractionation. The cytosolic isoenzymes can be specifically eluted from Gel Orange A by 1mM malate plus 1mM NADH, whereas the identical procedure does not remove the

isoenzyme from Percoll-purified mitochondria. The mitochondrial isoenzyme could not be eluted with 1mM oxaloacetate plus 1mM NAD^+ and was finally eluted in the presence of salt.

The homogeneous malate dehydrogenase from L. esculentum has an M_r of 76,000 and is a dimer containing sub-units of similar size. This compares with an M_r of 76,000 for the mitochondrial enzyme from the cotyledons of Citrullis vulgaris, which is also a dimer containing sub-units of similar size (Walk et al., 1969).

Fractionation Studies

Evidence from the sub-cellular fractionation experiments has established unequivocally that organic acid metabolism takes place in the mitochondria. There is no evidence to suggest that the glyoxylate cycle is operative in mature green or ripe tomato fruit, as citrate synthase was not found in the microbody fraction. All the enzymes under study were exclusively mitochondrial with the exception of malate dehydrogenase, which was located both in the cytosol and the mitochondria but was not detected in the microbody fraction.

Mitochondria purified from tomato fruit during all stages of development maintained their integrity (in terms of enzyme latency) for days rather than hours. It is highly significant that citrate synthase activity diminished by less than 3% from PPM which had been stored at 4°C for seven days. These mitochondria had been extracted from mature green fruit, and in a crude isotonic solution over the same time period, the specific activity of citrate

synthase would have fallen by 50%. This suggests that the complex interactions involved in the loss of enzymic activity are broken when the mitochondria are removed from the cell, and further suggests that the mechanism of loss is mediated other than by mitochondrial degradation.

The fractionation studies on three different cultivars corroborated the earlier results obtained from the whole fruit experiments, and the problems of fractionating red fruit were overcome using the storage facilities. The results highlighted an apparent difference between mature and immature fruit. Citrate synthase, malate dehydrogenase, NAD-dependent isocitrate dehydrogenase and NAD-dependent malic enzyme extracted from the mitochondria of mature green fruit exhibited not only a fall in activity but also a fall in specific activity, which, in the case of Sarina, was approximately 65% of the starting value. When the same enzymes were extracted from the mitochondria of mature green Marathon that had been stored in the modified gas atmosphere, the fall in specific activity was 50% and here, as in the whole fruit experiments, the specific activity was maintained at this new lower level. However, the enzymes extracted from the mitochondria of immature Marathon stored at ambient temperature displayed a 50% fall in activity but virtually no change in specific activity. To verify this result, a constant activity of 40 units of citrate synthase was loaded at each extraction onto the Percoll gradient, rather than a constant volume of crude resuspended mitochondria. In both cases the answer was identical: a fall in activity per gram of fresh weight but no fall in specific activity.

In summary, the data from the whole fruit experiments, supported by the findings of the fractionation studies, indicate a decline in specific activity of all enzymes of the citric acid cycle that were investigated during tomato fruit ripening. There appear to be two mechanisms involved:

1. In immature fruit there is a reduction in activity/g fresh wt of citrate synthase and malate dehydrogenase but little change in specific activity.
2. In ripening fruit that are picked at the mature green stage, there is a reduction in specific activity in addition to a reduction/g fresh wt.

This change in specific activity occurs (a) if the fruit are stored in a modified gas atmosphere, (b) if the fruit are stored in ambient conditions or (c) if the fruit are allowed to ripen normally on the vine. During the period of reduction in specific activities, there is a rapid increase in the specific activity of NADP-dependent malic enzyme (fruit stored in the gas atmosphere) and an increase in the concentration of glucose and fructose, and it is not until these changes have occurred that ethylene can be detected.

The questions now arise: what mechanisms might be responsible for what appears initially to be a form of coarse control of these enzymes, and what would be the function of such a control? It is considerably easier to formulate a hypothesis to explain the latter question, which is intimately involved with the phenomenon of senescence. It is possible to equate the terms maturation and ripeness with ageing and senescence when discussing the overall lifespan of tomato fruit, as ageing refers to the acquisition of

maturity with the passage of time, while ripeness, although normally associated with pleasant tactile and sensory experience, is in fact synonymous with deteriorative and senescent processes. Within the plant kingdom there are numerous examples of apparent programmed senescence (Leopold, 1980). Prominent amongst these is the synchronous death of the annual cereals. These plants are said to show a loss of survivorship by senescence and it is assumed that there is a form of temporal genetic mechanism which is responsible for the phenomenon. Within a species, climacteric fruit manifest similar survivorship curves, illustrated by the synchronous senescence of many fruit. The signal for the initiation of the process is not known but it is normally closely associated with the respiratory rise or the autocatalysis of ethylene. Physiologically, it makes sense to shut down central metabolism once the process of senescence has begun as, apart from a few specialised proteins, energy is not required for as many biosynthetic purposes. The data presented in this thesis suggest that this may happen in the case of tomato fruit, where the specific activities of several of the citric acid cycle enzymes fall to approximately 40% of their pre-climacteric values. This phenomenon may prove to be an early symptom of programmed senescence. The possible mechanisms involved can now be discussed.

(a) Since similar results in terms of enzyme activity are obtained either by removing mature green fruit from the vine or allowing them to ripen normally on the vine, it is possible that an operon-based system may be involved and that the derepressor is supplied via the plant. Removal of fruit from the vine, or the formation of an abscission layer within the petiole, would

effectively remove the flow of this compound(s) to the fruit. The problem with this hypothesis is that it implies a rapid turnover rate for these enzymes, as 50-60% of activity is lost within 72h. However, as shown in this thesis, if mitochondria are purified by Percoll fractionation and stored at 4°C for a week, very little activity is lost. The implication is that in addition to the cessation of enzyme synthesis, there is the initiation of a discriminatory degradative mechanism. This latter mechanism is obviously absent in immature fruit. Immature fruit contain approximately twice the amount of protein/g fresh wt of mature fruit and removal from the vine either halts completely or seriously disrupts protein synthesis. In immature fruit removed from the vine, the activities of citrate synthase and malate dehydrogenase fall slowly for several weeks. However, it is probable that the normal turnover mechanism is operative, as there is a corresponding slow loss of protein, with the net result that there is little change in specific activity.

(b) An interesting point illustrated by the data is the extent of the fall in specific activities of these enzymes. These values do not drop to zero but stabilise at approximately 60% below the starting values. It is possible, that this could be accounted for by post-translational modification. Modification systems are well known e.g. adenylylation, uridylylation, phosphorylation and thiol modification; however, any system would involve some form of trigger mechanism such that, as the fruit ripen, the enzymes would be modified to 40% efficiency. An

alternative proposal is that these enzymes are coded on multiple gene copies and that 60% of the genes are switched off. This argument is tenable if the selective degradative mechanism described in (a) is invoked, or if turnover is extremely rapid.

(c) A further possibility, and one that cannot be discounted, is a loss of mitochondria. This argument is weakened by two points: 1. The observation that it is not only mitochondrial enzymes that exhibit changes in specific activity but also cytosolic malate dehydrogenase. 2. The well established fact that, in many fruit, mitochondria remain functionally intact long after the climacteric peak has occurred, and when degeneration has begun in all other cellular organelles (Bain and Mercer, 1964; Simpson et al., 1976). How mitochondrial integrity is maintained at a time when total capacity drops to low levels is not clear (Frenkel et al., 1968), especially since 80–90% of mitochondrial proteins are nuclear coded. Ozelkok and Romani (1975) studied the longevity of mitochondria isolated from pear and avocado. They measured the capacity of mitochondria to survive in vitro by their ability to retain respiratory control and they concluded that in neither tissue did the capacity to 'survive' alter during ripening. The data from the present study support the findings of Ozelkok and Romani (1975). In terms of enzyme latency and respiratory control there appeared to be little difference between mitochondria extracted from mature green and red fruit. It may well be that the majority of mitochondrial proteins have slow turnover rates which would enhance the longevity of the organelles in these circumstances and make the selective degradative mechanism more plausible.

It would be quite possible to explore all the mechanisms discussed here. Substantial purification of the citric acid cycle enzymes would be necessary. Antibodies raised to these enzymes could subsequently be used to quantify the amount of enzyme protein present at different stages of ripening. This would allow determination of these enzyme levels independent of activity measurements and may indicate whether the enzymes are inhibited by modification or proteolytic breakdown. Enzyme level, however, is a balance between turnover and de novo synthesis and a sharp fall could be accounted for by a decrease in synthesis. This could be measured utilising one of the modern protein synthesising systems to translate the required mRNA. The products could then be determined using antibodies.

If, as the data suggest, a novel form of coarse control of the citric acid cycle and related enzymes occurs in tomato fruit, it can be described as one of the earliest symptoms of senescence. Further experimentation is needed together with studies on other fruit, to determine if this is a general phenomenon. If this is found to be so, and, if the control is mediated through 'temporal' genes, then our understanding of senescence in plants will be enhanced. The future manipulation of such a system to reduce dark respiration and thereby possibly increase crop yield cannot be discounted.

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